

**REGULATION OF OBESITY-INDUCED ADIPOSE TISSUE
INFLAMMATION AND INSULIN RESISTANCE THROUGH
MODULATION OF IMMUNE CELL FUNCTION**

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirement for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

The recruitment of immune cells into adipose tissue is a hallmark of obesity and a key event contributing to the development of adipose tissue inflammation and insulin resistance. However, the mechanisms regulating functions of immune cells in adipose tissue niches in response to obesity are poorly understood. In the present studies, I have demonstrated that hematopoietic-specific microRNAs and a functional cytokine, interferon tau, act as critical regulators for activation of adipose tissue resident immune cells including macrophages and B cells which subsequently affect obesity-induced tissue inflammation and insulin resistance.

First, miR-150KO mice display exacerbated obesity-induced tissue inflammation and insulin resistance. This phenotype is recapitulated by the adoptive transplantation of isolated miR-150KO B cells from obese mice into mice lacking B lineage cells. Further, miR-150KO B cells exerted potent stimulatory effects on both T cells and macrophages. Several miR-150 target genes were identified and knockdown of those genes individually or in combination alleviated B cell actions upon stimulation by altering important pathways downstream of the B cell receptor complex.

Second, I have shown that miR-223 is a critical mediator for PPAR γ action in controlling M2 activation, and subsequent beneficial effects that mitigate tissue inflammation and insulin resistance. Impaired PPAR γ functions

were observed in miR-223KO mice with respect to suppression of inflammation and insulin sensitivity. Disruption of miR-223 in macrophages impaired PPAR γ -dependent M2 activation, whereas overexpression of miR-223 further enhanced PPAR γ -mediated M2 activation. miR-223 is a PPAR γ -induced gene during M2 activation as confirmed by chromatin immunoprecipitation analysis and luciferase reporter assays. Two new miR-223 target genes *Rasa1* and *Nfat5* are crucial regulators of M2 activation via the PPAR γ -miR-223 axis.

Third, using a diet-induced obese mouse model, I discovered enhanced insulin sensitivity in obese mice administered IFNT compared to control mice, and this was accompanied by a significant decrease in secretion of proinflammatory cytokines and an increase in M2 macrophages in adipose tissue. Further studies revealed that IFNT is a potent regulator of macrophage activation that favors M2 responses.

Collectively, the findings from these thesis studies open a window to the development of novel therapeutic strategies to mitigate obesity-related diseases.

ACKNOWLEDGEMENTS

I would like to express my greatest gratitude to my mentors, Drs. Fuller W. Bazer and Beiyan Zhou, for their invaluable guidance, trust, and encouragement during my Ph. D. program at Texas A&M University. Thanks to Dr. Bazer for introducing me to various fields of research, especially reproduction, obesity, immunology, nutrition, and hematopoiesis. I appreciate Dr. Zhou for her mentorship on my research projects and her efforts in my scientific training. The most important things I have learned from Dr. Zhou are the passion and enthusiasm for science and the braveness for scientific exploration, and I believe these will pave the way for my future academic career. I also would like to give my deep appreciation to Dr. Guoyao Wu for encouraging me to join the Ph. D. program at Texas A&M University and his invaluable advice on my research projects. Dr. Wu is also one of the greatest teachers I have met. I am grateful for the great help and comments from Drs. Stephen Safe and Robert Chapkin on preparation of my manuscripts. I also thank Dr. Kathrin Dunlap for her advice on my projects involving reproductive biology.

I also appreciate the advice and assistance of other students including Srikanth Kanameni, Tyler Brehm, Richard Cheng-An Chang, Haiqing Wang, Hui Song, Andrew Morin, Patali S. Cheruku, Alexander Tseng, Alzahra Alshayeb, Xiaoqiu Wang, Jing Xu, Xiaoling Zhu, Ashley Keith, Will Frank, Michael Uzelac, Vijayalekshmi Nair, and Karen Triff for their help and assistance. Without their

assistance, it would have been impossible for me to accomplish the research projects described in my dissertation.

Finally, I would like to thank my family for providing the greatest support in my life and for always standing with me to face any challenge.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Introduction

Insulin resistance is the key etiology contributing to the development of metabolic syndrome.¹ A prolonged state of metabolic syndrome contributes to the development of type 2 diabetes mellitus (T2DM) that is also a major cause of various complications such as stroke, heart diseases, kidney failure, and blindness. Over the past several decades, obesity is now an epidemic and has become one of the most common causes of insulin resistance. In parallel, there has been a dramatic increase in the incidence of T2DM. Indeed, in the United States, about 29.1 million people or 9.3% of the population have type 2 diabetes, and more than 80% of people with T2DM are overweight (BMI>25).²

As a hallmark of obesity, chronic low-grade tissue inflammation is one of the major contributors to the pathogenesis of insulin resistance.³⁻⁵ Other metabolic abnormalities such as adipose tissue hypoxia, lipotoxicity, endoplasmic reticulum stress and mitochondrial dysfunction ultimately converge in the development of tissue inflammation in the context of obesity.

Inflammatory signaling in adipose tissue

Visceral adipose tissues (VATs) are one of the major sites producing and releasing inflammatory cytokines, which extend beyond tumor necrosis factor α

(TNF α), interleukin 1 β (IL1 β), IL6, and interferon γ (IFN γ).^{3,4} The signaling pathways governed by c-Jun N-terminal kinases (JNK) and nuclear factor kappa B (NF κ B) exert profound impacts over production of these inflammatory cytokines by VATs in response to various stimuli (Figure 1.1).^{6,7} In response to the activation of toll-like receptors (TLRs) such as TLR2 or TLR4, the phosphorylation of the inhibitor of NF κ B (I κ B) leads to its dissociation from NF κ B.⁸ The released p65 translocate into the nucleus where it binds to DNA response elements that induce expression of inflammatory molecules including proinflammatory cytokines. The phosphorylated I κ B eventually undergoes degradation. In addition, adipocytes also express receptors for binding of inflammatory cytokines such as TNF α and IL1 β , which can activate the JNK signaling pathway. The phosphorylation of JNK triggers the activities of transcription factor activator protein 1 (AP-1)-mediated signaling that subsequently drives the inflammatory responses including production of proinflammatory cytokines. Thus, these transcription factors act in an autocrine manner to promote inflammatory responses in adipocytes. In contrast, adipocytes also secrete anti-inflammatory adipokines such as adiponectin that either directly suppress the activation of NF κ B signaling pathway or trigger peroxisome proliferator-activated receptor-gamma (PPAR γ) activation which represses production of inflammatory cytokines.⁹⁻¹¹ In the context of obesity, the increased levels of TLR4 ligands such as free fatty acid significantly enhance the activities of the inflammatory signaling pathways mediated by JNK and NF κ B in

adipose tissues and this is accompanied by an increase in the release of inflammatory cytokines.^{12,13} Furthermore, obesity-induced hypoxia dysregulates production of adiponectin from adipocytes, which causes hypoadiponectinemia and ultimately impairs the negative controls of the inflammatory responses.¹⁴

Mechanisms of inflammation-induced insulin resistance

Insulin serves as a nutrient sensing hormone exhibiting diverse functions such as stimulation of nutrient uptake by cells to maintain energy homeostasis. The actions of insulin vary in distinct metabolic tissues. For example, in skeletal muscle insulin stimulates the translocation of glucose transporter type 4 (GLUT4) to the plasma membrane that facilitates glucose uptake, whereas insulin resistance impairs glucose uptake by skeletal muscle.¹⁵ Insulin action in liver suppresses gluconeogenesis by down-regulating expression of key gluconeogenic enzymes.¹⁶ A reduction in insulin sensitivity in liver leads to increased hepatic glucose production.¹⁷ In adipose tissues, insulin has an anti-lipolytic function via decreasing hormone sensitive lipase activity, subsequently inhibiting free fatty acid release from adipocytes.¹⁸ In contrast, insulin resistance promotes the circulating concentrations of free fatty acids, which can decrease insulin sensitivity in skeletal muscle by activating the serine/threonine kinase, protein kinase C- θ (PKC θ).¹⁹

Insulin signaling involves a complex signaling cascade downstream of the insulin receptor.²⁰ Upon the binding of insulin, activation of the insulin receptor

enhances the phosphorylation of insulin receptor substrates 1-4 (IRS-1-4). The formation and activation of this insulin receptor complex activates the phosphatidylinositol 3-kinase (PI3K)-AKT and Ras-mitogen activated protein kinase (MAPK) pathways (Figure 1.1). The insulin-induced activation of the PI3K-AKT pathway leads to increased glucose uptake and suppression of gluconeogenesis, while the effects of insulin on mitogenesis and cell growth are mainly associated with the MAPK pathway activity. In addition, insulin stimulates cell growth and differentiation through activation of the PI3K-AKT and MAPK pathways. In addition to IRS-mediated insulin signaling, activation of insulin receptors also recruits and activates other insulin receptor substrates including src homology 2 containing protein (SHC) and growth factor receptor-bound protein 2 (Grb-2), which then triggers the Ras-MAPK signaling cascade.²¹ The phosphorylation of heterotrimeric G protein (Gαq/11) stimulates GLUT4-mediated uptake of glucose by activation of the PI3K-AKT signaling pathway.²² The formation of the adapter protein Cbl associated protein (CAP)/Cbl complex can stimulate translocation of GLUT4 to the cell membrane to facilitate glucose uptake.²³

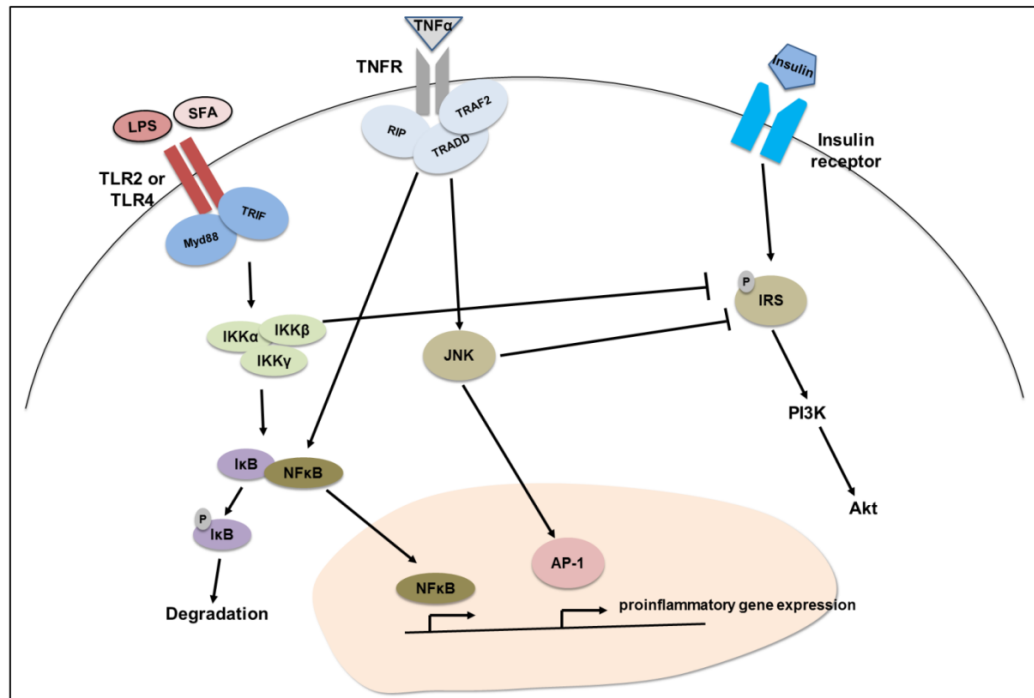


Figure 1.1 Inflammation signaling and insulin resistance. Activation of TLR2 or TLR4 and/or tumor necrosis factor receptor (TNFR) induces NFκB and JNK signaling. The serine kinases IKKβ and JNK can phosphorylate IRS1 and IRS2, which suppress downstream insulin signaling including the PI3K/AKT pathway. In addition, activation of IKKβ leads to phosphorylation and degradation of the inhibitor of NFκB (IκB) that subsequently permits translocation of NFκB to the nucleus. Similarly, activation of JNK results in the formation of transcription factor activator protein 1 (AP-1). Ultimately, both nuclear NFκB and AP-1 induce the expression of proinflammatory cytokines, which can contribute to the development of insulin resistance. TLR, toll-like receptor; Myd88, myeloid differentiation primary response gene-88; TRIF, TIR domain containing adaptor protein inducing IFNγ; RIP, receptor interacting protein; TRADD, TNF receptor-associated death domain; TRAF2, TNF receptor-associated factor 2; PI3K, phosphoinositide 3-kinase; NFκB, nuclear factor kappa B; JNK, c-Jun N-terminal kinases.

Chronic low-grade tissue inflammation is a hallmark of obesity and a well-known cause of obesity-induced insulin resistance through interrupting the insulin signaling in key metabolic organs such as white adipose tissue, liver and skeletal muscle. The inflammatory responses interrupt the insulin signaling pathway through several mechanisms in the context of obesity. For example, activation of JNK and I κ B kinase β (IKK β) can attenuate insulin signaling by directly inhibiting activation of the insulin receptor (Figure 1.1).^{24,25} In addition, the transcription factors NF κ B and AP-1 exert transcriptional control on the expression of genes that encode key components of the insulin signaling pathway including GLUT4, IRS-1, and AKT.²⁶⁻²⁹ Inflammatory cytokines, such as TNF α , trigger the signaling that also interferes with insulin action through modulation of various metabolic pathways that promote production of free fatty acids that decrease insulin sensitivity.^{30,31} Additionally, inflammation induces expression of the key enzyme sphingomyelinase for synthesizing ceramide that suppresses activation of AKT, that is downstream of the insulin signaling pathway.³¹

The link between tissue inflammation and metabolism

A key common denominator between metabolism and inflammation lies in the balance between intracellular lipids such as ceramides and sphingolipids. Holland et al. revealed that saturated fatty acid-induced activation of TLR4 signaling also up-regulates the biosynthesis of ceramide through activation of its

downstream component IKK β .³¹ However, under the stress of obesity, excessive activation of TLR4 signaling leads to the accumulation of ceramide in key metabolic tissues such as skeletal muscle, which impairs glucose uptake and eventually leads to insulin resistance.³¹ In contrast, knockout of TLR4 prevents the abnormal biosynthesis of ceramide and obesity-induced metabolic syndrome.³¹

Interestingly, ceramide can activate adiponectin receptors and adiponectin promotes ceramidase activity and modulates the balance between ceramides and sphingosine-1-phosphate.³² These regulatory impacts of adiponectin on ceramide metabolism appear to be critical for multiple tissues, as it prevents apoptosis of cardiomyocytes and pancreatic β cells.³² In the context of obesity, the diminished level of adiponectin may account for the accumulation of ceramide.

Obesity-induced adipocyte hypoxia

During long-term excessive nutrient intake, adipocytes undergo hypertrophy and hyperplasia.³³⁻³⁵ In mice, the increase in adipose tissue mass is mainly due to the hypertrophy of adipocytes after 4 weeks of feeding a high fat diet (HFD), while prolonged HFD exposure leads to a significant increase in adipogenesis in epididymal adipose tissue, but minimal effects on adipogenesis in subcutaneous fat depots.³³ The expansion of adipose tissue mass enhances lipid metabolism and the fatty acid flux that stimulates activation of adenine

nucleotide translocase (ANT)-mediated uncoupling of respiration in adipocytes.³⁶ This increases oxygen consumption by adipocytes and induces relative cellular hypoxia that triggers expression of hypoxia-inducible factor 1 α (HIF1 α).^{36,37} Upon the up-regulation of HIF1 α expression in adipocytes, the production of chemokines such as chemokine (C-C motif) ligand 2 (CCL2) and leukotriene B4 (LTB4) increases the recruitment of immune cells into white adipose tissues, whereas loss of HIF1 α impairs secretion of these chemokine from adipocytes in response to hypoxia and prevents dramatic infiltration of immune cells.^{36,38} In addition, the transcriptional function of HIF1 α on gene expression requires the ubiquitously expressed subunit aryl hydrocarbon nuclear translocator (ARNT or HIF1 β) to form the heterodimer complex.³⁹ Interestingly, HIF2 α expression serves as the negative control to counteract HIF1 α function in adipocytes.^{40,41} There is a significant decrease in expression of HIF2 α in adipocytes and loss of HIF2 α in adipocytes promotes the inflammatory status of visceral fat depots in obese individuals.⁴¹ Thus, the hypoxia-induced HIF1 α expression in adipocytes acts as the early trigger for inflammation in white adipose tissue (Figure 1.2).

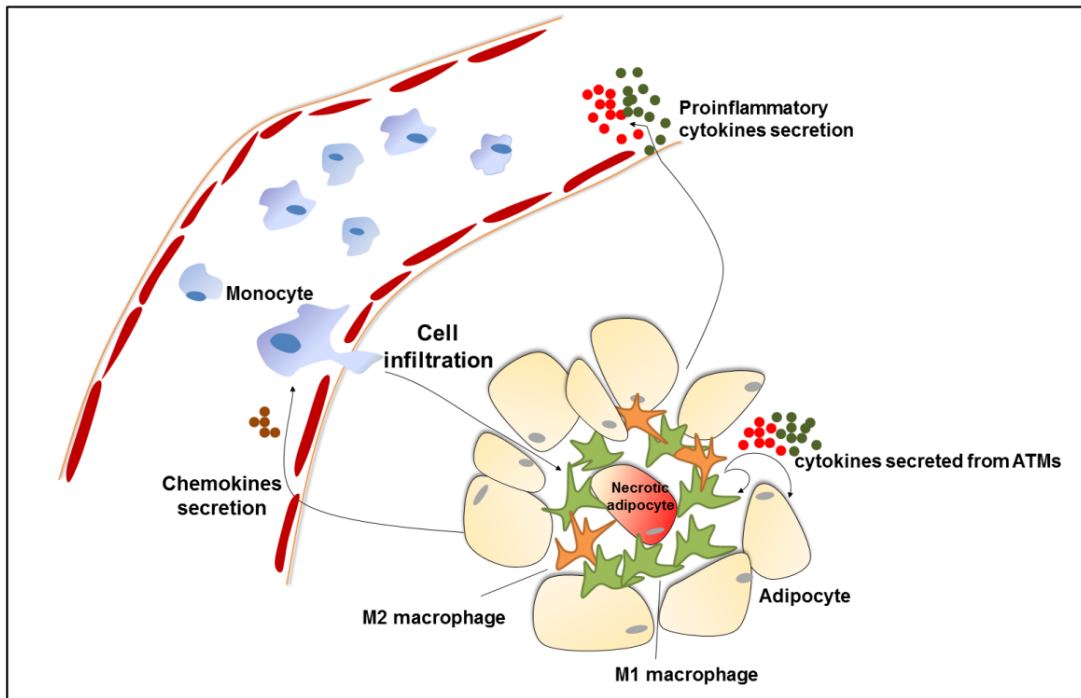


Figure 1.2 Macrophage recruitment into visceral adipose tissue. In the obese state, adipocyte hypertrophy and apoptosis lead to the production and release of chemokines that induce the recruitment of monocytes into visceral adipose tissue. In response to inflammatory stimuli, the infiltrated monocytes further differentiate into proinflammatory M1 macrophages, which also secrete proinflammatory cytokines and promote the inflammatory responses in both autocrine and paracrine manners.

Macrophage infiltration

In response to chemokines released from the white adipose tissue, circulating monocytes/macrophages are recruited into white adipose tissues and become a major immune cell component in this niche. The adipose tissue macrophages (ATMs) have crucial functions including the removal of dead adipocytes.^{42,43} Indeed, the infiltrated macrophages aggregate and surround the dying adipocytes to form “crown-like structures”. In the context of obesity, ATMs account for up to 50% of the stromal cell population in VATs and exert profound impacts on adipocyte functions.⁴¹

Among these chemokines, CCL2 can be produced by adipocytes and adipose tissue resident macrophages to exert profound effects on the recruitment of monocytes/macrophages into VATs (Figure 1.2).⁴⁴ The accumulation of white adipose tissues is accompanied by an increase in the circulating level of CCL2, due to the enhanced HIF1 α transcriptional functions.^{36,45} In the diet-induced obesity mouse model, the abundance of CCL2 in VATs increases within the first week of HFD feeding, and the secretion of this chemokine into plasma is elevated after 4 weeks of feeding a HFD.⁴⁶ In contrast, loss of CCL2 reduces the accumulation of ATMs, which eventually attenuates the obesity-associated adipose tissue inflammation and insulin resistance.⁴⁷ Ectopic expression of CCL2 from adipocytes is sufficient to enhance the accumulation of macrophages in VATs and to exacerbate insulin resistance of

mice on either a normal chow diet or a HFD.⁴⁸ The enhanced level of CCL2 facilitates the recruitment of macrophages into visceral fat depots through interacting with the chemokine (C-C motif) receptor 2 (CCR2) expressed on macrophages.^{49,50} In response to plasma CCL2, Geissmann et al. indicate that the peripheral blood monocytes expressing CCR2 are recruited into the inflamed tissues where they differentiate into macrophages that promote inflammatory responses.⁵¹ Lumeng et al. identified recruited macrophages in visceral fat tissues using an inert fluorescent dye PKH26 that is efficiently taken up by phagocytic cells in vivo.⁵² They further demonstrated that the recruited CCR2+ macrophages into VATs exhibit greater inflammatory responses compared to resident macrophages in the context of obesity.⁵² However, the CCR2 deficiency does not significantly affect infiltration of macrophages into adipose tissue in the context of obesity,⁴⁶ suggesting that there are other mechanisms involved recruiting these cells into the inflamed tissues. In addition, loss of CCR2 results in accumulation of undifferentiated myeloid cells that display inflammatory phenotypes in VATs during the time of feeding a HFD.⁴⁸

Recently, chemoattractant leukotrienes (LTs) have been identified as mediators controlling macrophage recruitment into white adipose tissue under obesity-induced stress.⁵³ LTs are proinflammatory lipid mediators that can be rapidly released from activated leukocytes to promote migration of leukocytes into tissues.^{54,55} Adipocytes are a major cell type secreting this chemoattractant, and its expression in adipocytes isolated from VATs is up-regulated by leptin

during obesity.⁵³ The elevated levels of LTs contribute to recruitment of macrophages into VATs, whereas LT-deficient mice accumulate fewer ATMs in response to feeding a HFD.⁵³ Thus, LTs play critical roles in regulating macrophage chemotaxis in the context of obesity. In response to LTs, circulating CD11b⁺ monocytes express the G protein-coupled receptor BLT-1 that is also induced in response to the HFD feeding.^{55,56} BLT-1 plays a critical role in the functions of LTs in inducing macrophage infiltration, as evidenced by decreased accumulation of ATMs in obese BLT-1-deficient mice.⁵⁶ In addition, knockout of BLT-1 prevents increases in proinflammatory M1 ATMs, but not M2 responses under obesity-induced stress, which ultimately attenuates the inflammatory status in white fat tissue of obese BLT-deficient mice.⁵⁶

Macrophage polarization

Adipose tissue macrophages exert profound impacts on homeostasis and functions of white adipose tissues through their polarization. The activation pattern of ATMs covers a full spectrum of activation stages between well-defined classical (M1) and alternative (M2) activation statuses in response to distinct microenvironmental cues.⁵⁷ In response to T helper type 1 (Th1) cytokines, lipopolysaccharide (LPS) or free fatty acids, M1 macrophages display classical proinflammatory responses depending on TLRs and activation of NFκB/JNK signaling pathways.^{58,59} This cell signaling cascade leads to the release of a set of proinflammatory cytokines, such as TNFα and IL1β, which directly impair the

insulin signaling pathway and activate the inflammatory responses of adipocytes through paracrine mechanisms.^{13,60} Recently, an epigenetic landscape at cytokine encoding gene loci for these proinflammatory cytokines was established during M1 activation.⁵⁷ Activation of TLR releases epigenetic "brakes" such as B-cell CLL/lymphoma 6 (BCL6) from gene loci and concomitant induction or activation of demethylases, and removal of inhibitory histone marks H3K27me3 and H3K9me3.⁶¹⁻⁶³ In addition, the ATP-dependent complex BAF (also known as SWI/SNF) is required for remodeling nucleosomes to facilitate recruitment of signaling transcription factor NFκB, and to increase activation histone marks such as H3K4me3, and activation of Pol II, ultimately inducing expression of proinflammatory cytokines.^{64,65} In contrast, the response to Th2 cytokines such as IL4 and IL13 by M2 macrophages leads to production of anti-inflammatory cytokines such as IL10 through the recruitment of PPARγ or other transcription factors.^{66,67} In addition, activation of PPARγ-dependent cell signaling leads to suppression of the inflammatory phenotype through blocking activation of NFκB signaling pathways. Activated PPARγ also induces expression of M2 responses-related genes which involve fatty acid metabolism and mitochondrial activities.⁶⁸⁻⁷⁰ The IL4-induced activation of STATs promotes expression of the histone demethylase JMJD3 that directly binds M2 genes such as arginase 1.⁷¹ Conversely, histone deacetylase 3 (HDAC3) acts as a "brake" repressing IL4-induced M2 responses by deacetylating putative enhancers of

M2 genes.⁷²⁻⁷⁴ Thus, histone methylation and acetylation are essential for the expression of M2 activation-associated genes.

For animals in the lean state, ATMs are preferentially activated as M2 phenotypes and express genes such as IL10 and arginase 1, which promote tissue repair and angiogenesis.⁵² In the context of obesity, ATMs primarily exhibit proinflammatory M1 phenotype in VATs in response to the proinflammatory cytokines and chemoattractants.^{52,75} Lumeng et al. demonstrated that resident macrophages in adipose tissue of lean animals undergo M2 activation while most macrophages infiltrating into VATs exhibit M1 phenotypes during the course of animals becoming obese.⁵² In addition, the infiltrated M1 macrophages preferentially locate around the dying adipocytes that form “crown-like structures”, and M2 ATMs reside in interstitial spaces between adipocytes.^{52,75} The proinflammatory cytokines secreted by M1 ATMs act in an autocrine or paracrine manner to enhance the inflammatory status of macrophages and adipocytes in visceral fat tissues of obese individuals.^{60,75,76} In contrast, in a conditional cell ablation system mediated by diphtheria toxin receptor to deplete CD11c+ cells, there is a significant decrease in accumulation of M1 ATMs in VATs which is accompanied by a decrease in inflammatory responses in adipose tissues and eventually improved insulin sensitivity of obese mice.⁷⁷ Given the critical roles of NF κ B/JNK in triggering the inflammatory responses in ATMs, knockout of IKK β or JNK suppresses M1 polarization which

is sufficient to attenuate the inflammatory status of white adipose tissue under obesity-induced stress.^{13,78,79}

Besides visceral fat tissues, macrophage polarization has critical roles in the development of inflammation in other key metabolic tissues such as liver and skeletal muscle.⁸⁰ Similar to adipose tissue, liver also has resident macrophages, termed Kupffer cells, which display a non-inflammatory phenotype and play critical roles in maintaining homeostasis of the liver.⁸¹⁻⁸³ Under obesity-induced stress, there is a remarkable increase in the recruitment of CCR2+ macrophages into liver in response to increases in the chemokine CCL2, and these infiltrated macrophages primarily exhibit proinflammatory M1 activation that promotes the inflammatory status in liver.⁸⁴ The transplantation of CCR2-deficient bone marrow cells into WT mice results in less accumulation of macrophages in liver compared to mice that received CCR2+ bone marrow cells after being fed a HFD which demonstrated that recruitment of macrophages into liver is dependent on CCR2 expression.⁸⁴ Furthermore, the recruited macrophages promote accumulation of hepatic triglycerides due to the up-regulation of expression of genes required for fatty acid synthesis and triglyceride esterification in the context of obesity.⁸⁴ In the skeletal muscle, some studies suggest that macrophage infiltration occurs in the small intermuscular adipose depots and that most of these cells exhibit a proinflammatory phenotype which impairs insulin signaling in skeletal muscle cells and their uptake of glucose.^{85,86} However, Bruun et al. and Tam et al. reported that skeletal muscle

has no significant increase in macrophage recruitment.^{87,88} Nevertheless, increases in proinflammatory cytokines impair activation of the insulin signaling pathway in skeletal muscle.^{59,85-88}

Regulation of adipose tissue macrophage polarization

Given the significant contribution of ATM polarization to inflammatory responses in white adipose tissue, it is critical to understand the networks governed by various transcription factors and epigenetic regulators in controlling the activation of ATMs. For example, TLR4-signaling induces expression of interferon regulatory factor 4 (IRF4) which exerts inhibitory functions for activation of proinflammatory M1 ATMs.⁸⁹ This is critical to maintain a balance in the activation status of ATMs in response to various stimuli in the microenvironment. In contrast, loss of IRF4 exacerbates M1 responses of ATMs to feeding a HFD, which ultimately promotes insulin resistance.⁹⁰ The IL4-activated signaling also induces expression of IRF4 required for expression of a set of M2 response-associated genes.⁷¹ The histone demethylase Jumonji domain-containing protein 3 (JMJD3) removes the inactivatory H3K27me3 marks at the *Irf4* locus and facilitates expression of this transcription factor in response to IL4 stimulation.⁷¹ Other transcription factors, kruppel-like factor 4 and 6 (KLF4 and KLF6), members of the zinc finger family, also have profound impacts on macrophage polarization.^{91,92} Upon stimulation with Th2 cytokines, such as IL4 or IL13, activation of signal transducer and activator of transcription

6 (STAT6) in macrophages induces expression of KLF4 that is critical for M2 responses.⁹² Ectopic expression of KLF4 increases the abundance of PPAR γ that is accompanied by increased expression of M2 activation-associated genes such as arginase 1, mannose receptor (Mrc1), resistin-like α (Retnla), and chitinase 3-like 3 (Chi3l3).⁹² The expression of KLF4 is suppressed in M1 macrophages upon LPS stimulation, whereas overexpression of KLF4 impairs the M1 activation phenotypes, due to its suppression of activities of NF κ B by attenuating the recruitment of the coactivators p300 and p300/CBP-associated factor (PCAF).⁹² In contrast, KLF4 deficiency enhances NF κ B transcriptional activity by increasing recruitment of these cofactors.⁹² Thus, KLF4 is critical for suppressing inflammatory responses in macrophages. In the context of obesity, there is a significant decrease in the abundance of KLF4 in the stromal vascular fraction of VATs in obese individuals, suggesting that the suppression of KLF4 expression facilitates activation of M1 ATMs.⁹² In contrast to the roles of KLF4 in macrophage polarization, KLF6 promotes an M1 phenotype and suppresses M2 responses. The expression of KLF6 is induced by stimulation of ATM with IFN γ or LPS, but suppressed in response to IL4 and IL13. KLF6 promotes expression of M1 activation-associated genes including TNF α , IL1 β , COX2, and MIP-1 α in macrophages, whereas macrophages without KLF6 show attenuated expression of these genes.⁹¹ KLF6 also has suppressive effects on the M2 phenotype induced by IL4, as evidenced by suppression of M2 activation-related genes such as arginase 1 and PPAR γ in macrophages with overexpression of KLF6.⁹¹

Further studies showed that activation of KLF6 enhances the recruitment of the NF κ B co-activator p300 to the promoter of genes encoding proinflammatory cytokines in macrophages upon LPS stimulation.⁹¹ KLF6 also acts as a repressor of M2 responses by suppressing actions of PPAR γ .⁹¹ Thus, the activation-specific expression of KLF6 plays critical roles in macrophage polarization.

In addition to transcriptional networks, microRNAs (miRNAs) exert an additional layer of regulation to control macrophage polarization (Figure 1.3). Similar to the expression pattern of key transcription factors during macrophage polarization, the myeloid-specific miRNAs are differentially expressed in response to distinct stimuli and exert profound impacts on the macrophage activation processes. Our recent study demonstrated that miR-223 is a critical regulator of ATMs polarization.⁹³ IL4-induced cell signaling dramatically increases expression of miR-223 in macrophages; however, its expression is not significantly changed in response to stimulation with LPS. The high abundance of miR-223 in macrophages favors M2 responses, whereas a deficiency in miR-223 decreases expression of PPAR γ and arginase 1 in IL4-induced M2 macrophages. In addition, macrophages lacking miR-223 are hypersensitive to LPS stimulation, as evidenced by the improvement in expression of costimulatory markers CD69 and proinflammatory cytokine TNF α . Furthermore, the regulatory functions of miR-223 in M1 macrophages involve its negative control of expression of a pro-diabetic gene, Pknox1. In the context of obesity,

miR-223-deficient ATMs preferentially exhibit proinflammatory M1 responses and a less anti-inflammatory phenotype than WT ATMs. Bone marrow transplantation of miR-223KO ATMs increases the inflammatory status in visceral fat depots and subsequent insulin resistance in obese recipient mice fed a HFD which suggests that miR-223-regulated macrophage polarization has significant effects on adipose tissue functions. Another microRNA, let-7c, has similar expression patterns and functions as miR-223 in macrophage polarization.⁹⁴ Ectopic expression of let-7c in LPS-induced M1 macrophages diminishes the inflammatory responses including the expression of proinflammatory cytokines IL12 and surface marker major histocompatibility complex class II (MHC-II). In addition, the LPS-stimulated macrophages show a phenotypic switch from M1 to M2 after overexpression of let-7c. Conversely, knockdown of let-7c in IL4-induced macrophages results in transition from an M2 to an M1 phenotype and a reduction in M2 responses, suggesting a critical role of let-7c in maintaining M2 responses. Further, the mechanism by which let-7c regulates macrophage polarization is related to its suppression of expression of target gene CCAAT/enhancer binding protein δ (C/EBP δ) that is a critical regulator of inflammatory responses of macrophages.

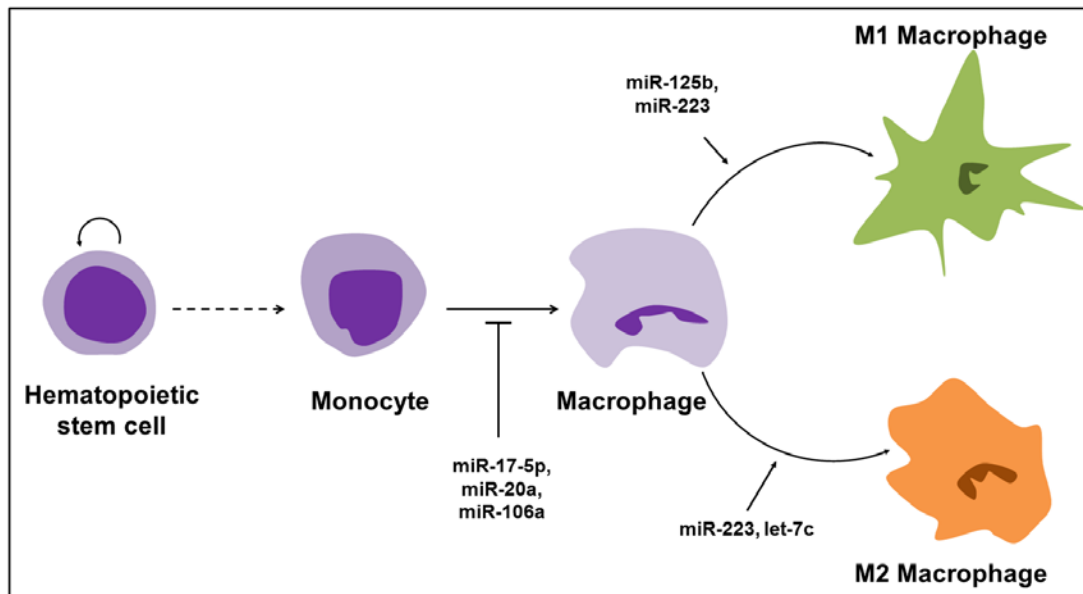


Figure 1.3 microRNA regulation of macrophage polarization in adipose tissue. microRNAs exert profound impacts on the differentiation or polarization of macrophages. For example, microRNAs such as miR-17-5p, miR-20a, and miR-106a repress the differentiation from monocyte to macrophage. miR-223 and let-7c favors M2 macrophage activation, while miR-125b is a positive regulator for the M1 responses.

In contrast to the suppressive effects of miR-223 and let-7c on M1 activation, miR-125b is a positive regulator of M1 macrophage activation.⁹⁵ Macrophages have increased expression of miR-125b upon IFN γ stimulation, accompanied by increased expression of costimulatory markers CD69, CD80, and CD86. In addition, overexpression of miR-125b further enhances expression of these surface markers of macrophages in response to IFN γ stimulation. Furthermore, transcription factor IRF4, a negative regulator of M1 activation, is the target gene of miR-125b in regulating macrophage activation. Therefore, it is

critical to understand the regulatory networks governed by transcription factors or/and miRNAs in controlling the polarization of ATMs.

Other innate immune cells in adipose tissue

Emerging evidence has shown that macrophages are not the only immune cells contributing to the development of tissue inflammation in the context of obesity (Figure 1.4). Neutrophil recruitment from blood to the inflamed tissue is a hallmark of early innate immune responses to the secreted chemoattractants LTB₄ and IL8.⁹⁶ Elgazar-Carmon et al. reported a significant increase in infiltration of neutrophils into VATs 3 days after initiating HFD feeding; however, the population of visceral fat tissue neutrophils did not change after 7-days of feeding a HFD.⁹⁷ Talukdar et al. found that recruited neutrophils exert profound effects on insulin signaling in white adipose tissue and liver through secreting pathogenic neutrophil elastase.⁹⁸ The production of neutrophil elastase increases during the course of obesity. Deletion of neutrophil elastase promotes insulin sensitivity of liver, as evidenced by the enhanced ability of insulin to inhibit hepatic glucose production and reduce concentrations of free fatty acids in blood compared to WT mice. In addition, there is an increase in insulin-stimulated phosphorylation of Akt in white fat depots after depletion of neutrophil elastase. Further, neutrophil elastase exhibits suppressive effects on insulin action in hepatocytes and adipocytes through degradation of IRS-1, and increased TLR4-mediated signaling and macrophage recruitment, which

contributes to the development of inflammation and insulin resistance. A recent study reveals that production of the endogenous inhibitor of neutrophil elastase, serine protease inhibitor α 1-antitrypsin (A1AT), is significantly reduced in livers under the stress of obesity due to obesity-induced leptin resistance.⁹⁹ The imbalance between neutrophil elastase and A1AT leads to tissue inflammation and insulin resistance in the context of obesity. In contrast, overexpression of A1AT or administration of a neutrophil elastase inhibitor can protect individuals from obesity-associated metabolic abnormalities. Taken together, neutrophils are key regulators of tissue function in the context of obesity.

Resident eosinophils in white adipose tissue are the major cells secreting IL4 to sustain M2 activation of ATMs.¹⁰⁰ Along with M2 ATMs, the proportion of eosinophils in adipose tissue is negatively correlated with adiposity in mice due to the reduction of innate lymphoid type2 (ILC2) cells that secrete IL5 and IL13 to recruit eosinophils.^{101,102} In addition, the ILC2 cells induce production of chemokines CCL11 and CCL24 from adipocytes to recruit eosinophils. Eosinophil deficient mice have low numbers of M2 macrophages in VATs and subsequent insulin sensitivity. Conversely, increases in eosinophils in adipose of IL5 transgenic mice induce M2 activation of ATM and protect them from obesity-induced insulin resistance. These studies provide strong evidence for critical roles of eosinophil in maintaining metabolic homeostasis in white fat depots. Given the critical roles of ILC2 cells in recruiting eosinophils, loss of ILC2 cells leads to a decrease in the population of adipose tissue eosinophil and

subsequent M2 ATM content that eventually compromises insulin sensitivity of adipose tissue.¹⁰¹ These characteristics mirror the phenotype of eosinophil-deficient mice. Thus, eosinophils play a role in the maintenance of M2 polarization in adipose tissue in obese individuals via secretion of anti-inflammatory cytokines IL4 and IL13.

Mast cells are critical regulators of allergic responses and tissue homeostasis and remodeling through secreting groups of proinflammatory and immunomodulatory molecules such as histamine, cytokines, chemokines, and proteases. Given that the development of obesity involves extracellular matrix remodeling and angiogenesis, significant increases in the recruitment of mast cells into white adipose tissues is accompanied by expansion of adipose tissue mass.^{35,103} Liu et al. demonstrated that mast cells residing in white fat pads exhibit important functions with respect to energy expenditure and glucose homeostasis in the context of obesity.¹⁰³ Using a mast cell-deficient mouse model, their results suggest that mast cells contribute to obesity-induced expansion of adipose tissue mass by stimulating expression of proteases and promoting angiogenesis in white adipose tissues. Further, adoptive transfer of cytokine-deficient mast cells revealed that IL6 and IFN γ secreted by mast cells induce expression of the cysteine protease cathepsin in adipose tissue. In addition, pharmacological stabilization of mast cells efficiently protects mice on a HFD from obesity-induced metabolic syndrome. Thus, mast cells may link inflammation to tissue remodeling in white adipose tissue in obese individuals.

Functions of adipose tissue T cells

In addition to the innate immune responses, the adoptive immune cells such as T lymphocytes located in adipose tissue exert profound impacts on the metabolic functions of adipose tissue (Figure 1.4). The resident CD3+ T cells in adipose tissue account for about 10% of the stromal vascular fraction in lean mice,¹⁰⁴ and there are two main groups of CD3+ T cells dependent on their phenotypic expression of the surface co-receptor CD4 or CD8. Further, CD4+ T cells in adipose tissues can be subcategorized into proinflammatory Th1 and Th17 cells and anti-inflammatory regulatory T (Treg) and Th2 cells.

In the context of obesity, the VATs are also characterized by an increase in the accumulation of IFN γ -secreting Th1 cells and a diminished population of Th17 cells.¹⁰⁴ The tissue-specific lymphocyte accumulation mainly depends on the MHC-antigen conjugate. Winer et al. demonstrated that T cells in adipose tissue undergo selective pressure in the complementarity determining region 3 (CDR3) region to generate T cell receptors (TCRs) that recognize specific antigens in fat.¹⁰⁴ This suggests that the expansion of T cells in VATs is driven in an antigen-specific manner in the context of obesity. Interestingly, there is no significant change in the accumulation of CD4+ T cells in subcutaneous fat depots during the development of obesity.¹⁰⁴ This may be due to the great polyclonal repertoire of CD4+ T cells residing in subcutaneous adipose tissues.^{104,105} Although the ATMs are antigen-presenting cells,¹⁰⁶ some studies

found that T cell expansion in VATs precedes changes in the population of ATM.^{104,107,108} A recent study provides evidence that adipocytes enhance activation of the MHC II pathway to facilitate expansion of CD4+ T cells in VATs during the early stages of development of obesity.¹⁰⁹ After 2 weeks of a HFD, expression of the adipokine leptin increases and stimulates production of IFN γ by resident CD4+ Th1 cells to induce the expression of MHC II in adipocytes of mice.¹⁰⁹ In addition, adipocytes of VATs exhibit greater expression of MHC II compared to that for subcutaneous fat depots which may account for the lack of change in CD4+ T cells in subcutaneous fat.¹⁰⁹ Knockout of MHC II in adipocytes decreases CD4+ T cells in adipose tissues,¹⁰⁹ suggesting critical roles for expression of MHC II by adipocytes in controlling proliferation and activation of CD4+ T cells. Furthermore, Th1 cells act as positive regulators of adipose tissue inflammation through MHC II-mediated IFN γ production and activation of M1 ATMs.^{108,109} In contrast, MHC II-deficient mice show reduced accumulation of CD4+ T cells in VATs, accompanied by decreased IFN γ production and the proportion of M1 ATMs under the stress of obesity.^{109,110}

In contrast to the increased expansion of proinflammatory Th1 cells, there is a decreased the proportion of adipose tissue resident Treg cells that exert anti-inflammatory effects in the context of obesity.^{104,111} Similar to Th1 cells in adipose tissue, Treg cells that reside in VATs have a unique T cell receptor repertoire for the recognition of specific antigens compared to Treg cells in the lymph nodes.¹⁰⁵ In addition, Cipolletta et al. reported that activation of the

PPAR γ -dependent signaling pathway is critical for controlling the accumulation and phenotype of Treg cells residing in adipose tissues.¹¹² The obesity-induced decrease in production of IL4 that can trigger PPAR γ action may account for the decreased population of Treg cells in adipose tissue in the context of obesity. Given its anti-inflammatory function, resident Treg cells in adipose tissue attenuate the inflammatory status. Depletion of Treg cells significantly exacerbates adipose tissue inflammation and insulin resistance under obesity-induced stress.¹¹¹ Conversely, expansion of Treg cells in adipose tissue by injection of IL2 and IL2-specific monoclonal antibody prevents obesity-associated metabolic abnormalities in mice.¹¹¹ Restoration of Treg cells in VATs by CD3-specific antibody promotes insulin sensitivity in obese mice.¹⁰⁴

In addition to CD4⁺ T cells, CD8⁺ T cells accumulate in VATs in the context of obesity.¹⁰⁷ However, there is no significant change in the population of CD8⁺ T cells in subcutaneous fat depots.¹⁰⁷ Jiang et al. demonstrated that the expression of CD11a, a component of lymphocyte function antigen-1 (LFA-1) expressed on T cells, plays a critical role in the accumulation of CD8⁺ T cells in VATs through interaction with intercellular adhesion molecule-1 on adipocytes.¹¹³ Indeed, the population of CD11a^{high}CD8⁺ T cells increases in VATs during the course of development of obesity, whereas obese mice depleted of CD11a have fewer CD8⁺ T cells resident in VATs compared to obese WT mice.¹¹³ Nishimura et al. suggested that the infiltration of CD8⁺ T cells into VATs occurs within 2 weeks after mice are placed on a HFD, and the

number of CD8⁺ T cells reaches a peak after mice are fed a HFD for 15-weeks.¹⁰⁷ In addition, cytokines such as IL2, IL12, and IL18 secreted by cells in adipose tissue of obese mice promote proliferation and activation of CD8⁺ T cells.¹⁰⁷ Furthermore, CD8⁺ T cells are required for the infiltration of proinflammatory macrophages into adipose tissues obese individuals in response to the release of chemokine CCL2 during the early stages of obesity which is critical to the initiation of the inflammatory cascade in the adipose tissue depots.¹⁰⁷ Depletion of CD8⁺ T cells prevents increased recruitment of macrophages into adipose tissues and proinflammatory responses of ATMs in response to feeding a HFD and this is accompanied by attenuation of the inflammatory status of adipose tissues.¹⁰⁷

Given the significant impacts of T cells on the functions of VATs, it is critical to understand the regulatory mechanism for the activation of T cells. The transcriptional controls are essential to regulate immunological functions of T cells in response to various cues in the microenvironment. As discussed earlier, Treg cells in adipose tissue exhibit a distinct phenotype such as a T cell receptor repertoire that differs from that of their counterparts in spleen and lymph nodes.¹⁰⁵ Cipolletta et al. provided evidence that PPAR γ has specific transcriptional control of gene expression patterns in Treg cells that are resident in VATs compared to the PPAR γ functions in Treg cells in lymph nodes.¹¹² Gene expression profiling revealed that PPAR γ actions promote expression of genes related to leukocyte migration and activation in resident Treg cells in VAT, but

not Treg cells in lymph nodes. Furthermore, enhanced PPAR γ activation increases expression of chemokine receptor CCR2 and a key transcription factor forkhead box P3 (FOXP3), that is accompanied by increased accumulation and activation of Treg cells in VATs under the stress of obesity.¹¹² The activated Treg cells exert their immunoregulatory functions primarily through transcriptional networks controlling production of the anti-inflammatory cytokine IL10. For example, in response to the inflammatory conditions, the production of IL10 from Treg cells requires activation of transcription factor PR domain containing 1, with zinc finger (ZNF) domain (PRDM1, also known as BLIMP1) that is induced by the IRF4-dependent cell signaling pathway.¹¹⁴ In addition, PRDM1 acts via a negative feedback loop to limit accumulation of Treg cells in tissues by suppressing expression of pro-survival factor BCL2 and chemokine receptor CCR6.¹¹⁴

Recent studies reveal that miRNA-mediated gene regulation represents a fundamental layer of post-transcriptional control of differentiation and activation of T cells. For example, miR-181a has profound functions in regulating TCR signaling that is critical for T cell development and homeostasis.¹¹⁵ Repression of miR-181a using specific antisense oligonucleotides dampens TCR sensitivity and impairs positive and negative selection of T cells in the thymus, whereas ectopic expression of miR-181a in mature T cells enhances their sensitivity to peptide antigens.¹¹⁵ miR-181a can directly suppress the expression of phosphatases that decrease TCR signals, including protein tyrosine

phosphatase type 11 (Ptpn11), Ptpn22, dual-specificity protein phosphatase 5 (Dusp5), and Dusp6, thereby lowering the TCR engagement threshold for cellular responses.¹¹⁵ Another miRNA, miR-146a, is induced following TCR engagement and serves as an important feedback regulator of NFκB signaling that is a downstream component of the cascade for TCR signaling.¹¹⁶ Further study revealed that miR-146a prevents the hyper-responsive inflammatory response in activated T cells through targeting NFκB signal transducers TNF receptor-associated factor 6 (Traf6) and IL1 receptor-associated kinase 1 (Irak1).¹¹⁷ Zhao et al. observed that miR-146a-deficient mice have spontaneous myeloproliferation and myeloid cell tumorigenesis due to loss of the miR-146a-dependent feedback loop.¹¹⁸ However, the regulatory networks in controlling differentiation and activation of resident T cells in adipose tissue to various stimuli have not been elucidated.

Function of adipose tissue B cells

Given the essential role of B cells in adoptive immune responses, adipose tissue resident B cells exert profound impacts on the function of adipose tissues in response to the alterations in cues in the microenvironment. B cells are originally generated in bone marrow, and mature B cells are released from spleen into the bloodstream. The chemokine chemokine (C-X-C motif) ligand 13 (CXCL13) is one of the critical mediators of B cell migration into tissues where they encounter antigens and undergo activation.¹¹⁹ Interestingly, the recruitment

of circulating B cells into VATs occurs shortly after the initiation of feeding a HFD, and the population of B cells reaches a peak 3 to 4 weeks later to become one of the major immune cells populations in white fat depots (Figure 1.4).^{110,120} Winner et al. suggested that activation of adipose tissue B cells increased immunoglobulin class switching to IgM+IgD- or IgG+ phenotypes, especially to pro-inflammatory IgG2c under the stress of obesity.¹¹⁰ This increase in pathogenic IgG antibodies directly interrupts insulin signaling in key metabolic tissues such as liver and skeletal muscle through interactions with antigenic targets that are associated with the endoplasmic reticulum stress.¹¹⁰ In addition, adipose tissue B cells promote MHC-dependent activation of CD4+ and CD8+ T cells in adipose tissue niches which contributes to increased production of pro-inflammatory cytokines such as IFN γ and subsequent induction of activation of M1 macrophages.¹¹⁰ Mice without mature B cells have reduced activation of proinflammatory T cells and macrophages in VATs and improved insulin sensitivity under the stress of obesity as compared to obese WT controls.¹¹⁰ In contrast, the adoptive transfer of IgG antibodies isolated from HFD-fed WT mice exacerbates insulin resistance of HFD-fed B-cell-deficient mice in a Fc-dependent manner, suggesting that IgG antibodies are mediators of B cell function in regulating insulin sensitivity in the context of obesity.¹¹⁰ In contrast to the pathogenic IgG antibodies, a recent study suggests that B cells in adipose tissue produce and secrete more proinflammatory cytokines such as IFN γ and IL6, but less anti-inflammatory IL10 in obese compared to lean control mice.¹²¹

This obesity-induced alteration in cytokine expression patterns by B cells in adipose tissue increases the Th1/Th17 to Treg cell ratio in white fat depots leading to adipose tissue inflammation and insulin resistance.¹²¹ However, obese mice without mature B cells accumulate more Treg cells compared to the WT mice on a HFD, indicating the critical role of B cells in regulating T cell homeostasis in adipose tissue in the context of obesity.¹¹⁹ However, mechanisms regulating expression of cytokines by B cells in adipose tissue and their impact on the adipose tissue T cell homeostasis have not been elucidated. In addition, like T cells, B cells are not homogeneous, and consist of distinct subsets including B1 and B2 cells, which differ in their location and function. B1 cells are enriched in mucosal tissues and pleural/peritoneal cavities, and these cells preferentially undergo T-cell independent activation when encountering antigens. B2 cells accumulate in secondary lymphoid organs such as lymph nodes and spleen, and mainly respond to T-dependent antigens responsible for adoptive humoral immunity. In the context of obesity, both B1 and B2 cells are recruited into VATs, but their roles in this niche have not been elucidated.

Besides B1 and B2 cells, there is a group of IL10/transforming growth factor β (TGF β)-secreting regulatory B (B_{reg}) cells that reside in adipose tissue to prevent excessive and pathological inflammatory responses. Nishimura et al. reported that the adipose tissue B_{reg} cells are characterized by their constitutive production of IL10 through activation of PI3K/Akt signaling in response to the release of fatty acids from adipocytes.¹²² However, the spleen or bone marrow B_{reg} cells do not express IL10 under basal physiological conditions. The C-X-C chemokine receptor type 4 (CXCR4) ligand CXCL12 released from adipose tissue contributes to the recruitment of IL10-producing regulatory B cells into adipose tissues.¹²² In addition, a recent study by Wu et al. suggests that the spleen serves as a source of B_{reg} for the recruitment of these cells into the peritoneal cavity and VATs.¹²³ These IL10-secreting adipose tissue B_{reg} cells have suppressive effects on activation of proinflammatory CD8⁺ T cells and macrophages, as evidenced by an enhanced population of these cells in adipose tissues after mutation of IL10 expression in B_{reg} cells.^{122,123} In the context of obesity, the proportion of adipose tissue B_{reg} cells is diminished due to decreased production of CXCL12 by adipocytes of obese individuals and this eventually contributes to exacerbation of inflammation and insulin resistance.¹²² IL10 can also promote the viability of adipose tissue B_{reg} cells, whereas the reduction of IL10 in the context of obesity accounts for the decreased proportion of B_{reg} cells residing in adipose tissues.

The activation of B cells requires antigen-induced B cell receptor (BCR) signaling and signaling via cytokine receptors and co-stimulatory receptors. In response to distinct cues in the microenvironment, regulation of these complex processes during B cell activation involves regulatory networks governed by various mediators that include microRNAs. The microRNAs have critical roles in orchestrating the regulatory networks that activate B cells. For example, Mraz et al. recently reported that miR-150 activates B cell receptors by negatively controlling expression of GRB2-associated binding protein 1 (GAB1) and FOXP1. An abnormally high abundance of miR-150 in chronic lymphocytic leukemia significantly represses expression of GAB1 and FOXP1 and subsequently impairs the activity of B cell receptor signaling.¹²⁴ Another extensively studied microRNA in mature B cell is miR-155 which has significant effects on immune responses of B cells.¹²⁵ Mature B cells without miR-155 expression produced less IgG1 and cytokines such as TNF α . Further, miR-155 negatively controls expression of PU.1 that is a critical transcription factor downstream of the BCR signaling pathway in mature B cells.¹²⁵ However, the regulatory mechanisms for activation of B cells in adipose tissue has not been elucidated.

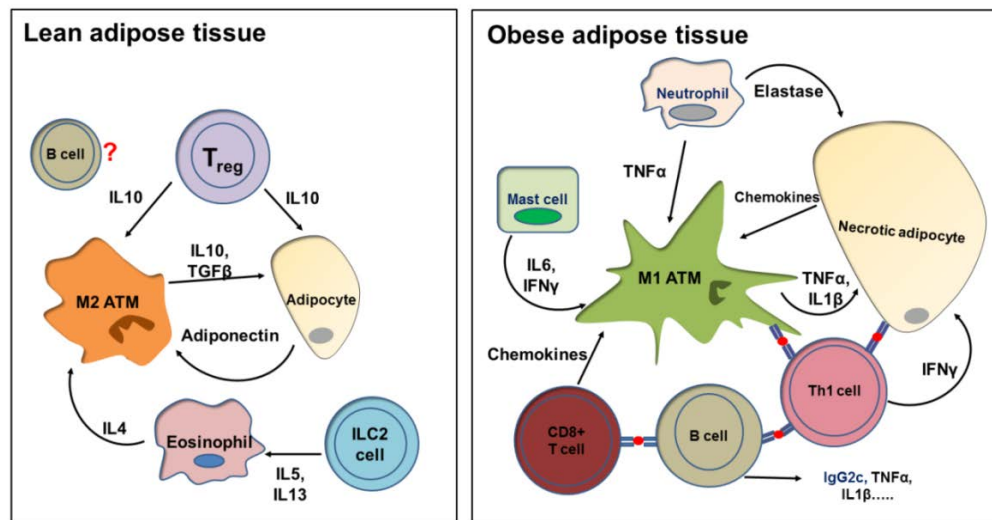


Figure 1.4 The interplay between immune cells and adipocytes in adipose tissue. In adipose tissue of lean mice, interleukin 5 (IL5) and IL13-producing innate lymphoid (ILC2) cells promote the maturation and recruitment of eosinophils that are the major source of IL4 in adipose tissue. Activated Treg cells respond to secrete anti-inflammatory IL10. Both IL4 and IL10 maintain the alternative M2 phenotype of adipose tissue macrophages (ATMs). In addition, adiponectin released from adipocytes contributes to the maintenance of the M2 phenotype of ATMs. In adipose tissue of obese individuals, adipocytes undergo cellular stress and secrete proinflammatory cytokines and chemokines which significantly elevate the population of proinflammatory M1 ATMs. Neutrophils also infiltrate into adipose tissues during the development of obesity and promote inflammation and insulin resistance by secreting tumor necrosis factor alpha (TNFα) and elastase. Mast cells can produce IL6 and interferon gamma (IFNγ) to promote activation of M1 ATMs. Macrophages and adipocytes activate Th1 cells in a major histocompatibility complex (MHC)-antigen manner. Adipose tissue resident B cells are also able to present antigens on MHC molecules to either CD8+ T cells or Th1 cells. In addition, B cells secrete pathogenic antibody IgG2c and proinflammatory cytokines. Thus, the interplay among these immune cells eventually contributes to insulin resistance.

PPAR γ agonists as anti-inflammatory therapies for insulin resistance

Given the critical roles of resident immune cells in tissue with respect to inflammation and insulin resistance in the context of obesity, therapeutic strategies to repress recruitment and activation of proinflammatory immune cells have potential importance.

PPAR γ signaling exerts profound effects that regulate activation of immune cells including M2 macrophages and Treg cells.^{66,112} Consistent with this notion, the antidiabetic thiazolidinediones (TZDs) including rosiglitazone and pioglitazone are PPAR γ ligands that promote activation of anti-inflammatory immune cells that reside in adipose tissues.^{66,112} For example, the TZDs exhibit robust anti-inflammatory effects and promote M2 responses in macrophages which contributes to the attenuated inflammation and insulin resistance in adipose tissue. Treatment with TZDs also enhances eosinophil numbers and activation of anti-inflammatory Treg cells in adipose tissue of obese individuals.^{112,126,127} In addition, PPAR γ acts as insulin sensitizer by triggering the PPAR γ -dependent transcriptional programs in various metabolic cell types.¹²⁶ Indeed, these drugs improve insulin sensitivity in key metabolic tissues including liver, adipocytes, and skeletal muscle.

Interferon tau: An anti-inflammatory cytokine

Interferon tau (IFNT) is secreted by mononuclear trophectoderm cells of ruminant conceptuses and was originally determined to be the pregnancy

recognition signal for establishment of pregnancy in ruminants such as sheep and cows.¹²⁸ IFNT is a member of type I interferon family, and this cytokine shares high amino acid sequence similarities with other type I interferons such as IFN alpha (IFNA) and IFN omega (IFNW).¹²⁹ IFNT also possesses antiviral, anti-proliferative, and immunomodulatory activities, with very low cytotoxicity even at high dosages.¹²⁹

Previous studies demonstrated the potential anti-inflammatory function of IFNT in various diseases models. For example, Soos et al. report that IFNT significantly prevented development of experimental allergic encephalomyelitis (EAE) through suppressing the expansion of proinflammatory CD4+T cells and production of TNF α following superantigen staphylococcal enterotoxin B (SEB) stimulation.¹³⁰ Results of a further study using the myelin basic protein (MBP)-specific TCR-transgenic mouse model suggests that IFNT promotes the production of IL10 and Th2 cytokines such as IL4 and IL5, but diminishes the secretion of Th1 cytokine IFN γ that attenuates EAE.¹³¹ In addition, Chaouat et al. observe that in vivo injection of recombinant ovine IFNT significantly enhances the production of IL10 and IL4 that is sufficient to prevent fetal resorption in mice having a CBA x DBA/2 placental genotype.¹³² Similarly, in pregnant sheep, IFNT limits the immune responses to the conceptus by suppressing the expression of MHC I and β 2 micoglobulin in uterine luminal and superficial glandular epithelia.¹³³ Using the non-obese diabetic mouse model, oral, intraperitoneal, or subcutaneous supplementation of IFNT delays the development of diabetes by

repressing pancreatic islet inflammation and increasing the number of splenic Treg cells.¹³⁴ Taken together, IFNT is a potential therapeutic treatment of obesity-induced tissue inflammation.

miRNAs and their therapeutic application in obesity

miRNAs are a group of highly conserved small noncoding RNAs (~22 nucleotides in length) that are produced by two RNase III proteins, Drosha and Dicer.¹³⁵ miRNAs function primarily through RNA silencing which is initiated by base pairing with their target mRNAs.¹³⁶ In this context, argonaute (AGO) proteins serve as effectors by recruiting factors that induce translational repression, mRNA deadenylation and mRNA decay. miRNA-binding sites are usually located in the 3' untranslated region (UTR) of their target genes. The domain at the 5' end of miRNAs that spans from nucleotide position 2 to 7 is crucial for target recognition and has been termed the 'miRNA seed'. The downstream nucleotides of miRNA (particularly nucleotide 8 and less importantly nucleotides 13-16) also contribute to base pairing with the target mRNAs. It is known that over 60% of human protein-coding genes contain at least one conserved miRNA-conserved miRNA-binding site and, considering that numerous non-conserved sites also exist, most protein-coding genes may be under the control of miRNAs.

Given the extensive regulation of miRNAs on protein-coding genes, it is not surprising that miRNAs also exert profound impacts on genetic networks and

subsequent physiological processes. Dysregulated expression of miRNAs in human and animal models are associated with various diseases and their progression, including obesity-associated metabolic syndrome that includes cardiovascular diseases.¹³⁷ However, how miRNAs regulate functions of tissue-resident immune cells such as adipose tissue resident B cells in the context of obesity is not well understood.

The recent discovery of extracellular miRNAs opens a new window to the understanding the functional mechanisms of this small non-coding RNA family. Interestingly, the expression patterns of extracellular miRNAs are correlated with various diseases, including type 2 diabetes and atherosclerosis, suggesting their potential roles as biomarkers for diagnosis of those diseases. Concentrations of miR-125a-5p, miR-126-3p, miR-221-3p and miR-222-3p in serum are reduced in patients with atherosclerosis compared to healthy subjects.¹³⁸ Extracellular miRNAs may present in different formats, including exosomal particles, microvesicles and argonaute-bound molecules.¹³⁷ However, major questions regarding their secretion and remote tissue targeting remain unanswered. For example, extracellular miRNAs can be passively released by damaged tissues and cells or be actively secreted by cells, acting as cell-cell communication factors. Further endeavors are necessary to address these questions so that translational therapeutic strategies can be developed and put into practice.

CHAPTER II

MIR-150 REGULATES INSULIN SENSITIVITY THROUGH MODULATION OF B CELL FUNCTION: A NOVEL PARADIGM FOR THE DEVELOPMENT OF INSULIN RESISTANCE

Resident B cells in adipose tissue account for 20% of the stromal cell population. However, their function and mechanisms of action in the adipose tissue niche are poorly elucidated. In this study, I report that miR-150, a crucial regulator for B cell formation and activation, is a potent modulator for obesity associated adipose tissue function by controlling activation of B cells and their cell-cell interactions with other immune cells. Mice with miR-150 deficiency display exacerbated obesity-associated tissue inflammation and systemic insulin resistance. This phenotype is recapitulated by the adoptive transplantation of isolated miR-150KO-B cells from obese mice into mice lacking B lineage (B^{null}), but not isolated immunoglobulin. Further, miR-150 deficient B cells exert potent stimulatory effects on both T cells and macrophages, ultimately leading to increases in inflammation and insulin resistance in adipose tissue. Several target genes of miR-150 have been identified and knockdown of these genes individually or together alleviated B cell actions upon stimulation by altering important pathways downstream of the B cell receptor complex. My results demonstrate a critical role for B cell function in adipose tissue, and that miR-150

and its regulatory molecular network are crucial regulators of both metabolic and immune homeostasis in the adipose tissue niche.

Introduction

Immune cells in adipose tissue orchestrate homeostasis of immunological and metabolic functions in adipose tissue.^{5,80,139-141} Significantly increased numbers of immune cells are crucial for the pathogenesis of obesity-induced chronic low grade inflammation, systemic insulin resistance, and subsequent high risk for cardiovascular diseases, type 2 diabetes, and metabolic syndrome.^{44,107,142-145} Among various immune cell lineages that infiltrate into adipose tissue of obese individuals, B cells become one of the major immune cells compared to their profiles in lean individuals, in both absolute numbers and relative proportion to visceral stromal cells.^{110,121,122} However, the function of adipose tissue resident B cells (ATBs) and their mechanisms of action have not been clearly elucidated.

In mouse models of obesity, the accumulation of B cells in visceral adipose tissues (VATs) reaches a peak 3 to 4 weeks after initiation of feeding a high-fat diet (HFD) and VATs become one of the major immune cell compartments in the adipose tissue niche.¹¹⁰ Results of recent studies suggest that B cells exert profound effects on adipose tissue homeostasis by influencing antibody production and cell-cell interactions with other immune cells.^{110,121} Importantly, B cells are heterogeneous, consisting of two distinct major subtypes,

B1 and B2, which are unique in their immune responses and interactions with other cells. For example, B1 cells, a major subtype of peritoneal B cells, exhibit more rapid immunological responses than B2 cells, but with a less-diverse and more poly-reactive antibody repertoire.¹⁴⁶⁻¹⁵⁴ Although the populations of both B1 and B2 cells increase in adipose tissue of obese individuals,¹¹⁰ the contribution of these B cell subsets to the pathogenesis of obesity-associated metabolic disorders remains poorly understood.

Previous studies, including ours, have demonstrated that miR-150 is a crucial regulator for B cell formation.^{155,156} Ectopic expression of miR-150 in hematopoietic stem cells specifically blocks B cell formation at the early progenitor stages;¹⁵⁵ whereas loss of miR-150 does not have a major impact on hematopoiesis except for enhancing the B1a cell sub-population relative to B2.¹⁵⁶ While accounting for less than 2% of cell types in blood and other major immune organs, B1a cells are a major B cell subtype in adipose tissue, composing 15% of the B cells.^{156,157} Therefore, I hypothesize that miR-150 regulates ATB cell activation and the communication between B cells and other immune cells and adipocytes.

In this study, we probed the function of ATB cells and their functional mechanisms of action using a transgenic mouse model, miR-150KO mice, along with adoptive transplantation assays. There is a specific increase in B1a cells in response to deletion of miR-150, whereas other cells in other hematopoietic cell

lineages are not affected. Using this unique miR-150 null mouse model we sought to understand the impact of B cells, specifically B1a cells, in regulating adipose tissue function in response to the stress of obesity, in particular, resident immune cell functions and cell-cell crosstalk in adipose tissue. Furthermore, we identified two new miR-150 target genes, *Elk1* and *Etf1* in this context and demonstrated that *Elk1*, *Etf1*, and a known target *Myb* are important mediators for miR-150 regulation of B cell activation. Thus, results of this study provides evidence to support a novel paradigm of microRNA regulation of ATB cell functions in obese-induced tissue inflammation and insulin resistance, and identifies new target genes for drug development to mitigate obesity-associated cardiovascular diseases and metabolic syndrome.

Materials and Methods

Animals

The generation of miR-150-deficient (miR-150KO) and B^{null} B6 (lack of mature B cells) mice has been described.^{156,158} Wild-type (WT) C57BL/6J mice were used as controls. All mice were maintained on a 12/12-hour light-dark cycle. Male mice 5 to 6 weeks of age were fed *ad libitum*. Mice were fed a HFD (Cat. No. D12492, Research Diets, Inc; Table A-1) or a low-fat diet (LFD; Cat. No. D12450B, Research Diets, Inc; Table A-2) for 12 weeks. After the feeding regimen, mice were subjected to phenotypic characterization and metabolic assays, including measurement of metabolic parameters in plasma, insulin and

glucose tolerance tests, and immunohistochemical analysis of tissues. All study protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Texas A&M University.

Flow cytometry analysis

Visceral stromal cells from visceral adipose tissue and splenic cells were stained with fluorescence-tagged antibodies ($0.125\ \mu\text{g}/10^6$ cells) to detect cell lineages. B cell subtypes were detected with antibodies against CD19 (Cat. No. 17-0193-82, eBioscience), CD5 (Cat. No. 11-0051-82, eBioscience) and CD43 (Cat. No. 12-0431-82, eBioscience); T cells were detected with antibodies against cell surface markers CD4 (Cat. No. 11-0041-82, eBioscience) and CD8 (Cat. No. 17-0081-82, eBioscience); myeloid cells were detected with antibodies against cell surface markers F4/80 (Cat. No. 11-4801-82, eBioscience), CD11b (Cat. No. 45-0112-82, eBioscience) and Gr-1 (Cat. No. 35-5931-82, eBioscience); macrophage subtypes were detected with antibodies against cell surface markers F4/80, CD11b, CD206 (Cat. No. 141706, Biolegend), CD11c (Cat. No. 12-0114-82, eBioscience). Macrophage activation was measured with antibodies against cell surface markers CD80 (Cat. No. 17-0801-82, eBioscience), CD69 (Cat. No. 45-0691-82, eBioscience), and CD86 (Cat. No. 11-0862-82, eBioscience). T cell activation was measured using antibodies against cell surface markers CD80, CD86, CD69, IFN γ (Cat. No. 48-7311-82, eBioscience), and IL2 (Cat. No. 12-7021-82, eBioscience). B cell activation was

measured using antibodies against cell surface markers CD80, CD86, CD69, IgA (Cat. No. 12-4204-82, eBioscience), IgM (Cat. No. 47-5790-82, eBioscience), and IgD (Cat. No. 17-5993-82, eBioscience). Data were analyzed using Flowjo or Accuri C6 (BD Biosciences) or Kluza (Beckman Coulter) software.

Immune cell isolation analysis

The T and B cells were isolated from VATs or spleen of wild type or miR-150KO mice (HFD or LFD). VATs were first mechanically dissociated and then subjected to collagenase II (2 mg/mL; Cat. No. 17101-015, Invitrogen; Table A-3) digestion for 30 min at 37°C. After lysing red blood cells (Table A-4), a single cell suspension was incubated with biotin-labeled antibodies against cell surface markers CD4 (Cat. No. 13-9766-82, eBioscience), CD8 (Cat. No. 13-0081-82, eBioscience) for T cells, B220 (Cat. No. 48-0452-82, eBioscience) for B cells or F4/80 for macrophages. Magnetic beads conjugated with streptavidin (Cat. No. 557812, BD Biosciences) were applied for cell isolation. Purity of each cell population was validated using flow cytometry before being subjected for further analysis (Figure 2.1A and B). Bone marrow-derived macrophages (BMDM) were prepared as previously described.¹⁶⁰ Macrophage maturation was examined by flow cytometry with antibodies against F4/80 and CD11b (Figure 2.1C).

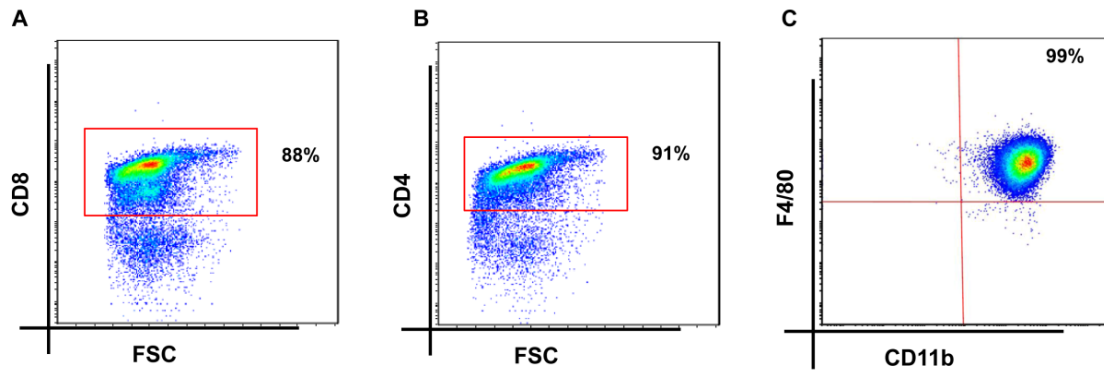


Figure 2.1 Immune cell isolation. Purified splenic CD8+ T cells (A) and CD4+ T cells (B) and bone marrow-derived macrophages (C) were analyzed by flow cytometry with antibodies against cell surface markers CD8, CD4, F4/80 and CD11b, respectively. FSC, forward scatter.

Immune cell activation analysis

In the B cell activation assay, naïve B cells from spleen were stimulated with lipopolysaccharide (LPS, 10 $\mu\text{g/mL}$) for T-cell independent activation or interleukin 4 (IL4, 10 ng/mL)/anti-CD40 (10 $\mu\text{g/mL}$; Cat. No. 14-0401-85, eBioscience) for T-cell dependent activation. The macrophage polarization assay was carried out following the protocol described previously.^{93,159} For macrophage polarization analysis, BMDMs were stimulated with LPS (100 ng/mL) for activation of M1 or IL4 (20 ng/mL) for M2 polarization. T cells were

activated by anti-CD3 (Cat. No. 16-0031-85, eBioscience) and anti-CD28 (Cat. No. 16-0281-85, eBioscience) pre-coated on a cell culture plate.

Activation of immune cells was examined by flow cytometry with antibodies against cell surface markers CD69, CD80, and CD86, Bioplex assays for cytokine production profiles, and quantitative RT-PCR for gene expression analysis. Concentrations of immunoglobulin including IgA (Cat. No. 88-50450-22, eBioscience), IgE (Cat. No. 88-50460-22, eBioscience), IgG1 (Cat. No. 88-50410-22, eBioscience), IgG2a (Cat. No. 88-50420-22, eBioscience), IgG2b (Cat. No. 88-50430-22, eBioscience), IgG2c (Cat. No. 88-50670-22, eBioscience), and total IgG (Cat. No. 88-50550-22, eBioscience) in plasma or B cell culture supernatant were measured using ELISA kits according to the manufacturer's instructions.

Adoptive transplantation assay

After 12 weeks on a high fat diet, spleens were collected from HFD WT and miR-150KO mice and mechanically dissociated. After lysing red blood cells (Table A-4), B220⁺ B cells from spleen were purified using anti-mouse CD45R/B220 (Cat. No. 13-0452-85, eBioscience) conjugated to magnetic particles (Cat. No. 557812, BD Biosciences). The purity of B cell was determined by flow cytometry with antibodies against cell surface marker B220 (Figure 2.2). A total of 1×10^7 B220⁺ B cells in 150 μ L of PBS were transplanted into each HFD B^{null} mouse via intraperitoneal (i.p.) injection. HFD B^{null} mice without B cell

transfer were controls. Cell engraftment in visceral fats and adjacent organs, as well as glucose tolerance were determined 2 weeks after the B cell injection.

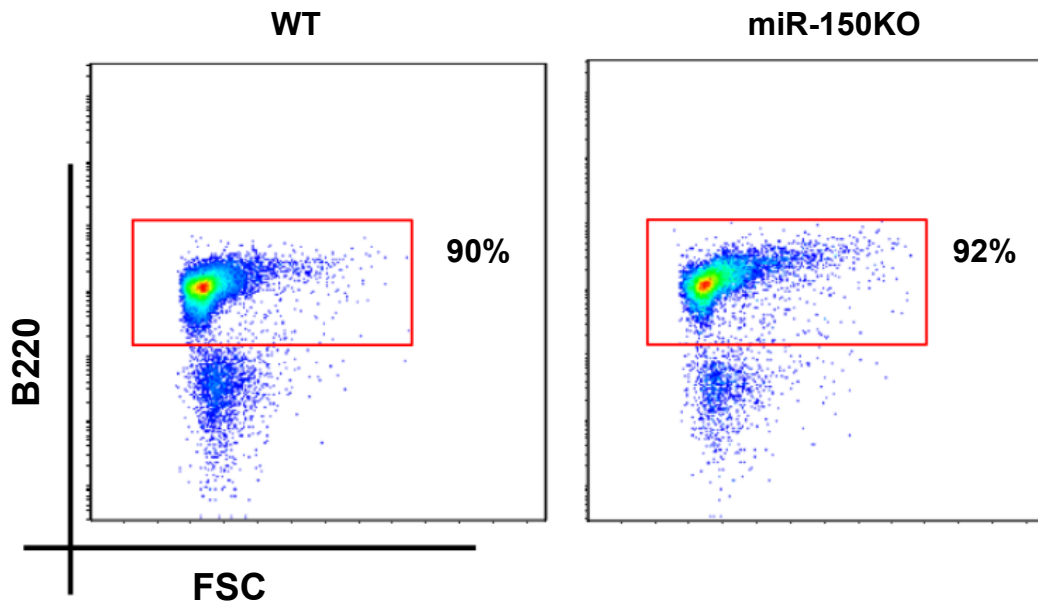


Figure 2.2 Isolation of splenic B cells for adoptive transplantation assay. B220+ B cells were purified from wild type (WT) or miR-150knockout (miR-150KO) mice fed a high fat diet using anti-mouse B220 conjugated magnetic beads. The results indicate that the about 90% of cells used for adoptive transplantation were B220+ cells. FSC, forward scatter.

Cell co-culture assay

The B cells isolated from spleens of WT or miR-150KO B cells were pre-activated with LPS (10 µg/mL), or IL4 (10 ng/mL)/anti-CD40 (10 µg/mL). After 48 h activation, pre-activated B cells were mixed with WT CD4⁺ or CD8⁺ T cells that were pre-activated using anti-CD3 (10 µg/mL) and anti-CD28 (10 µg/mL) pre-coated tissue culture plates at a ratio of 10:1. After activation for 96 h, pre-activated B cells were co-cultured in a trans-well plate with WT M1 (LPS, 100 ng/mL) or M2 (IL4, 20 ng/mL) BMDMs at a ratio of 1:10. The activation of co-cultured cells was examined as described in the section titled Immune cell activation analysis.

Isolation and injection of IgG

Immunoglobulin G (IgG) proteins were isolated from plasma of HFD-WT or miR-150KO mice using Dynabeads[®] Protein G (Cat. No. 10003D, Invitrogen). The IgG proteins were validated for their purify by gel electrophoresis (Figure 2.3). A total of 150 µg of IgG proteins in 150 µL of PBS were transferred into each HFD B^{null} mouse via i.p. injection on Day 0 and Day 3. HFD B^{null} mice without IgG protein transfer were controls. A glucose tolerance test was performed 1 week after injection of IgG.

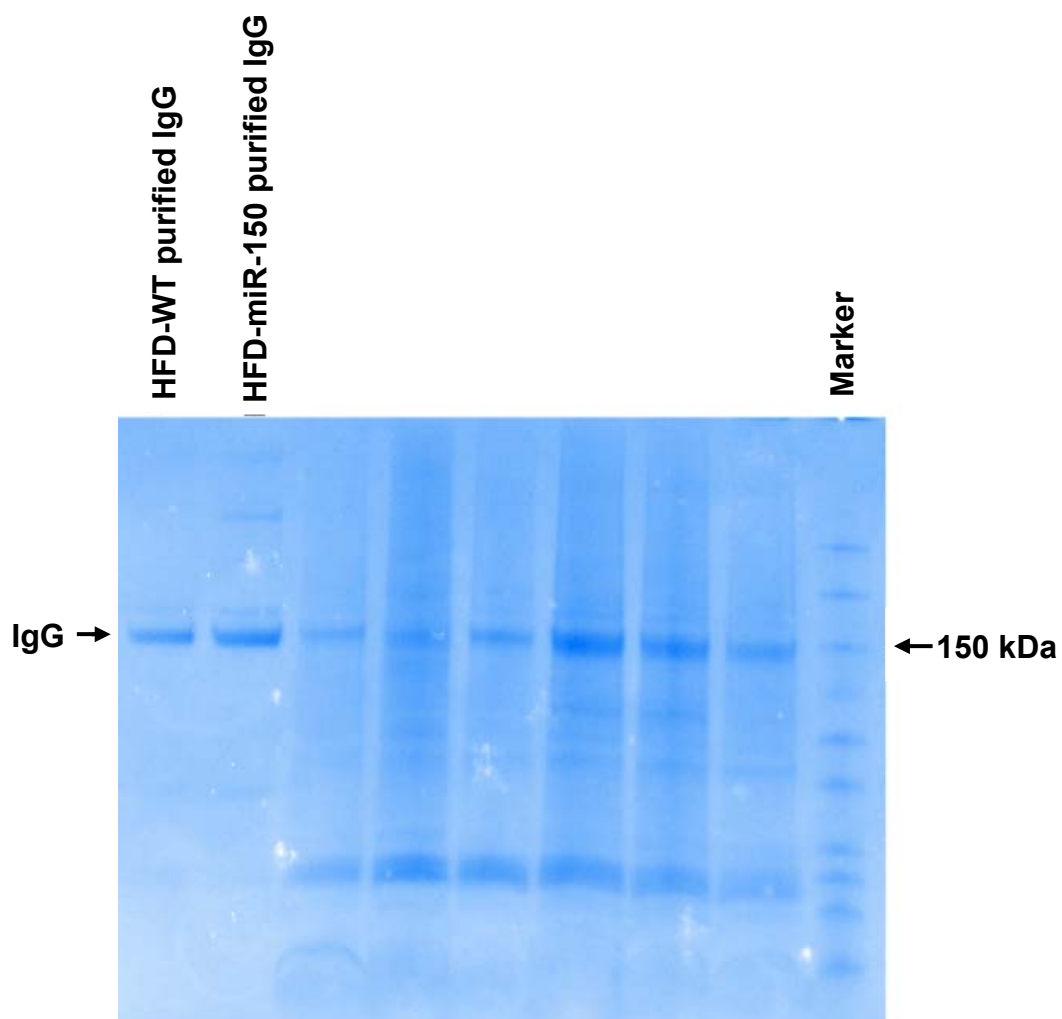


Figure 2.3 Immunoglobulin (Ig) G purification. IgG proteins were purified from plasma of wild type mice (WT) or miR-150knockout (miR-150KO) mice fed a high fat diet (HFD).

Luciferase reporter assay

The luciferase reporter assay was carried out following the protocol described previously.⁹³ Briefly, the full 3'-untranslated region (3'-UTR) sequence of the predicted target gene or at least the 250-bp flanking region of the predicted miR-150 binding site was cloned into the psiCheck2 Vector (Promega) downstream of the *Renilla* luciferase-coding region. The reporter constructs with oligonucleotides that mimic miR-150 or negative control oligonucleotides were cotransfected into HEK293 cells. At 48 h after co-transfection, the activities of *Renilla* luciferase were measured using the Dual-Glo luciferase reporter system (Cat. No. E2920, Promega) and normalized to the internal control firefly luciferase activity. Repressive effects of miR-150 on target genes were plotted as percentage repression in three biological assays that each contained three technical repeats.

Gene-specific knockdown with shRNAs

The pLKO.1-CMV-TurboGFPTM vector (Sigma-Aldrich) with inserted shRNAs (targeting *Myb*, *Elk1* or *Etf1*) was co-transfected with compatible packaging plasmids into HEK293T cells. The lentiviral supernatants were collected 72 h after transfection and used to infect purified splenic B220+ B cells. The empty vector was used as the control.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from adipose tissue B cells or BMDMs using the Trizol extraction protocol according to the manufacturer's instructions (Cat. No. R2050, Zymo Research). Gene expression analysis was performed using a iScript One-Step RT-PCR kit with SYBR Green (Cat. No. 170-8893, Bio-Rad) on Bio-Rad CFX384 (Bio-Rad). Data presented correspond to the mean of $2^{-\Delta\Delta Ct}$ from at least three independent experiments after being normalized to β -actin.

Bio-Plex protein expression assay

The concentrations of IL1 β , tumor necrosis factor- α (TNF α), IL6, IL10, and chemokine (C-C motif) ligand 2 (CCL2) in plasma were determined using a Bio-PlexTM cytokine assay (Cat. No. M60-009RDPD, Bio-Rad). Concentrations of insulin in plasma were determined using the Bio-Plex pro mouse diabetes insulin set (Cat. No. 171-G7006M, Bio-Rad). The abundance of total (Cat. No. 171-V60007M, Bio-Rad) and phosphorylated (Cat. No. 171-V50011M, Bio-Rad) c-Jun N-terminal kinases (JNK) in mature adipocytes were determined using the Bio-Plex cell signaling magnetic assays (Bio-Rad). These Bio-Plex assays were performed using the Bio-Plex MAGPIXTM multiplex reader (Bio-Rad). Results were analyzed using Bio-Plex Data ProTM software (Bio-Rad).

Statistical analyses

Results are expressed as means \pm SEM. Each data point derived from reverse transcription PCR assays represents an average of two technical replicates, and data were averaged over independently replicated experiments ($n = 3-4$ independently collected samples) and analyzed using the Student's t test. The overall group-effect was analyzed for significance using two-way ANOVA and Bonferroni post-test for each factor at each individual time. Data analyses were performed using Graphpad Prism version 6.0 software. A value of $P < 0.05$ was considered statistically significant.

Results

miR-150 deficiency exacerbates obesity-Induced systemic insulin resistance

Previous studies, including ours, demonstrated that miR-150 is a critical hematopoietic regulator that exerts profound effects on B cell formation.^{155,156} A miR-150 deficiency in mice enhances formation of B1 cells, a subtype of the B lineage that accounts for more than 15% of resident B cells in adipose tissue.^{156,157} To evaluate adipose tissue function under the stress of obesity, we challenged miR-150KO mice by feeding a HFD for 12 weeks. The miR-150KO mice on the HFD (HFD-miR-150KO) displayed no significant differences with respect to body weight gain or food intake compared to age-matched HFD wild type control mice (HFD-WT; Figure 2.4). However, HFD-miR-150KO mice

exhibited a significant increase in systemic insulin resistance and tissue inflammation as evidenced by results from glucose tolerance and insulin sensitivity tests (Figure 2.5A and B). The miR-150KO mice fed a LFD did not display abnormal concentrations of glucose or insulin in plasma during either the fasted or fed states compared to LFD wild type control mice. After 16 weeks on a the HFD, miR-150KO mice exhibited significantly higher concentrations of glucose and insulin in plasma, suggesting severe obesity-induced hyperglycemia and hyperinsulinemia conditions during both the fed and fasted states (Figure 2.5C and D). These results suggest that miR-150 is a crucial regulator for the development of obesity-induced insulin resistance.

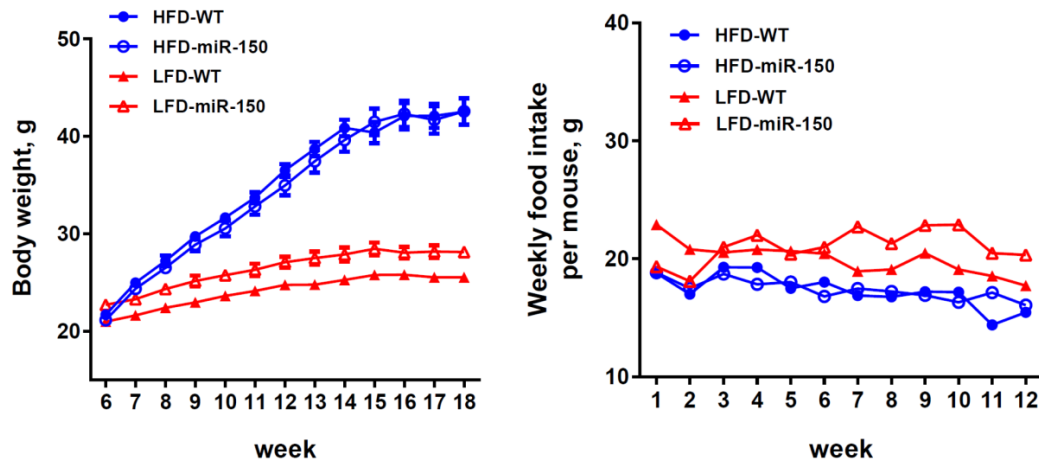


Figure 2.4 Body weight and food intake. During a 12-week feeding period on high fat (HFD) or low fat (LFD) diets, the body weight (grams, g) and weekly food intake (grams, g) of wild type (WT) and miR150 knockout mice were recorded weekly. The results indicate that miR-150 has no significant effects on the body weight and food intake of mice. Data are presented as mean \pm SEM (n=10).

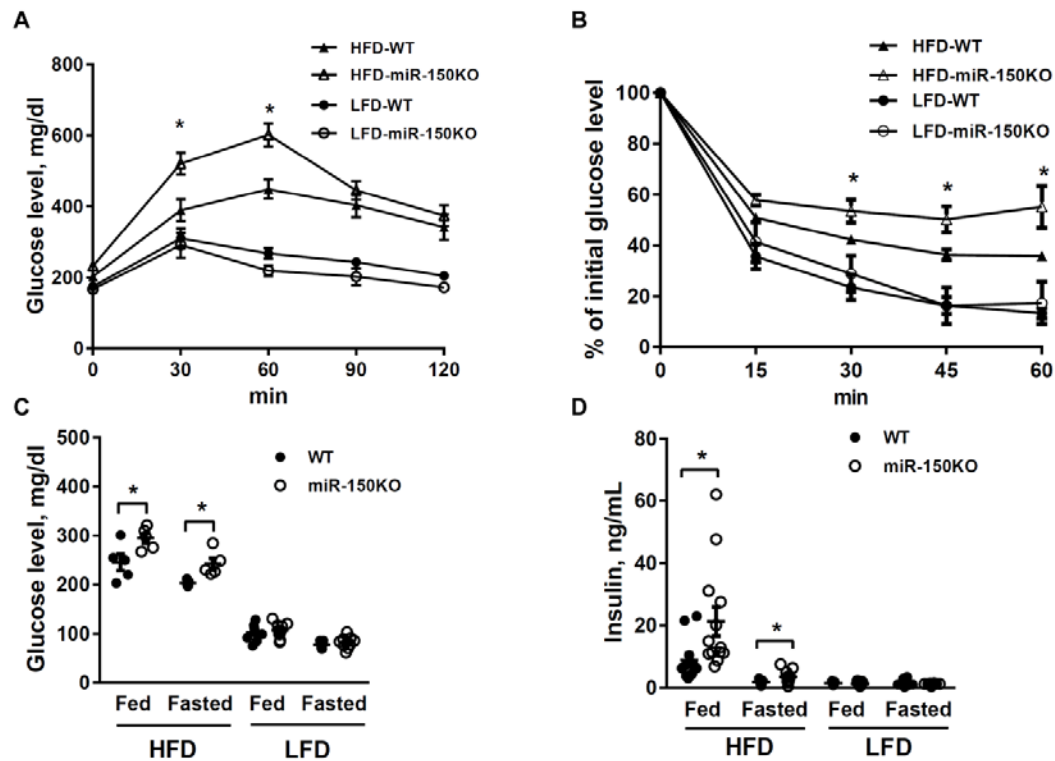


Figure 2.5 MicroRNA-150 (miR-150) ablation exacerbates obesity-induced systemic insulin resistance compared to that for wild type (WT) mice. A and B, Glucose tolerance test and insulin tolerance test after 12 weeks of feeding a high fat diet (HFD) or a low fat diet (LFD; $n=6-10$). The results indicated that miR-150 deficient mice showed exacerbated insulin resistance under obesity stress. C and D, Concentrations of glucose and insulin in plasma of miR-150 deficient (miR-150KO) mice or wild type (WT) mice fed a HFD, or fasted for 16 hours were greater than that of HFD-fed WT mice. However, miR-150 deficiency did not affect the glucose and insulin levels of LFD mice. Data are presented as mean \pm SEM. * $P<0.05$.

miR-150 deficiency promotes obesity-induced inflammation

Chronic adipose tissue inflammation is a major phenotype in obesity and a causal factor for obesity-associated insulin resistance.^{5,80,139} Given the metabolic abnormalities observed in HFD-miR-150KO mice, we examined the inflammatory features in adipose tissues. Although HFD-miR-150KO mice displayed a similar degree of adiposity and comparable size of adipocytes to HFD-WT mice based on histological analyses (Figure 2.6A), miR-150 deficiency significantly enhanced markers of obesity-induced adipose tissue inflammation. This included increased abundance of TNF α , IL1 β , and IL6 (Figure 2.6B), which was confirmed by findings of increased activities of c-Jun N-terminal kinases (JNK; Figure 2.6C) and nuclear factor- κ B (NF κ B) signaling pathways (Figure 2.6D) in VAT of HFD-miR-150KO as compared to the VAT of HFD-WT control mice. Concomitantly, we detected an increase in concentrations of inflammatory cytokines and a decrease in anti-inflammatory IL10 in plasma of HFD-miR-150KO mice (Figure 2.6E). In addition, HFD-miR-150KO mice had greater expression of CCL2 (Figure 2.6F) in VAT and increased concentrations of CCL2 in plasma compared to HFD-WT mice (Figure 2.6G). Compared to HFD-WT mice, the abundance of anti-inflammatory adiponectin was significantly less in VAT of HFD-miR-150KO mice (Figure 2.6H). Collectively, these results indicate that in the context of a miR-150 null genotype, there is a significant increase in obesity-associated systemic inflammation.

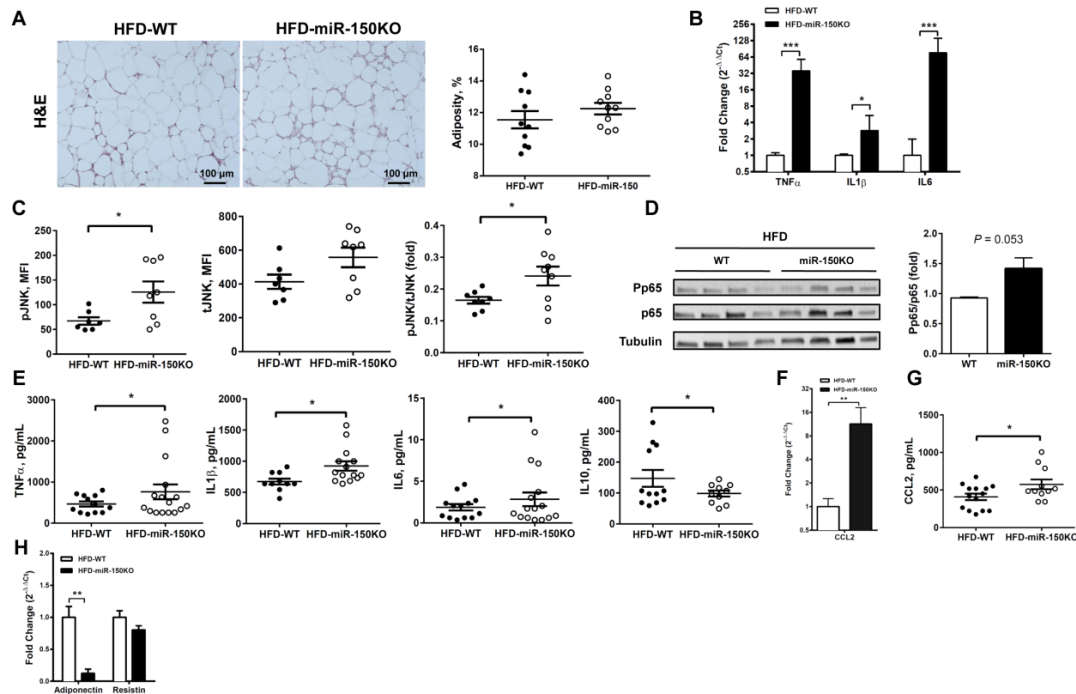


Figure 2.6 miR-150 regulates obesity-induced inflammation. A, Hematoxylin and eosin (H&E) staining of visceral adipose tissue (VAT) sections suggested similar size of adipocyte between wild type (WT) and miR-150 knockout (miR-150KO) mice. The adiposity was comparable among the mice on a high fat diet (HFD). B, miR-150 depletion resulted in increased expression of inflammatory cytokines tumor necrosis factor- α (TNF α), interleukin (IL) 1 β , and IL6 in VAT of mice fed a HFD (n=3). C and D, Loss of miR-150 led to elevated activation of c-Jun N-terminal kinases (JNK) and nuclear factor- κ B (NF κ B) signaling pathways in VAT of mice fed a HFD. MFI, medium fluorescence intensity. Western blots were performed with antibodies against p65 and phosphorylated p65 (Pp65). E, Concentrations of cytokines in plasma of mice fed a HFD were detected using the Bio-PlexTM Cytokine Assay (Bio-Rad). F and G, miR-150 deficient mice fed a HFD showed greater expression of chemokine (C-C motif) ligand 2 (CCL2) in VAT and concentration in plasma compared to the HFD WT mice (n=3). H, miR-150KO mice on a HFD had greater abundance of adiponectin but not resistin in VAT compared to HFD WT mice (n=3). Data are presented as mean \pm SEM. * P <0.05, ** P <0.001, *** P <0.0001.

miR-150 deletion alters adipose tissue inflammatory response and the proportion of B lymphocyte subtypes

To determine cell lineages that are major mediators of miR-150 actions in the context of obesity, we examined expression patterns of miR-150 in various hematopoietic and metabolically active tissues in lean mice. Consistent with previous results,¹⁵⁵ expression of miR-150 is high in most hematopoietic tissues, including lymph nodes, thymus and spleen, while low in white adipose tissues and skeletal muscle (Figure 2.7). Of note, the stress of HFD-induced obesity did not significantly alter the expression patterns of miR-150 in metabolic tissues, including liver, visceral fat depots, brown fat or muscle (Figure 2.8A). Given the crucial roles of immune cells in regulating obesity-associated insulin resistance and adipose tissue inflammation, we further evaluated expression of miR-150 in various adipose tissue cell compartments. As expected, mature adipocytes isolated from visceral fat expressed low amounts of miR-150 compared to mature B and T cells (Figure 2.8E). Interestingly, macrophages are a major immune cell population in stromal cells of adipose tissue in obese individuals,^{60,160,161} but they did not express high amounts of miR-150 as compared to lymphocytes (Figure 2.8E).

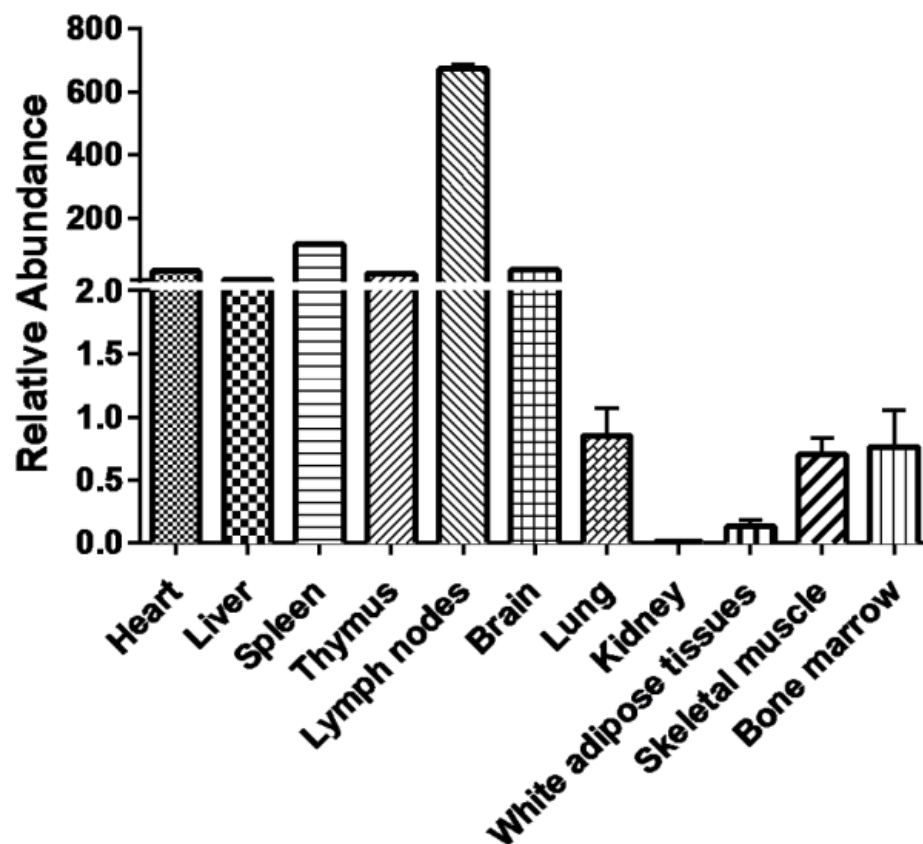


Figure 2.7 The expression of miR-150 in various tissues from lean wild type mice. The abundance of miR-150 in key hematopoietic or metabolic tissues of wild type lean mice was measured by reverse-transcription PCR analysis. The result indicated that hematopoietic tissues had high abundance of miR-150. Data are presented as mean \pm SEM (n=3).

Next, we examined the composition of immune cells in stromal cells isolated from VAT of HFD-miR-150KO and HFD-WT mice. Among all hematopoietic lineages examined, the proportion of CD4⁺ T cells, but not CD8⁺ T cells, was significantly higher in the miR-150 deficient than HFD-WT control mice (Figure 2.8B). Consistent with previous reports,^{93,160,162} macrophages are a major immune cell type in white adipose tissue of obese individuals that accounts for more than 30% of stromal cells (Figure 2.8B). Although total adipose tissue macrophages were comparable in HFD-miR-150KO and HFD-WT mice, miR-150 depletion resulted in more proinflammatory M1 macrophages (F4/80⁺CD11b⁺CD206⁻CD11c⁺, $P=0.002$, Figure 2.8C) in adipose tissues.

Given the high proportion of macrophages in adipose tissue niches and their contribution to the development of obesity-induced adipose tissue inflammation, we examined the impact of miR-150 ablation on polarization of macrophages. To exclude pre-exposure of in vivo adipose tissue to inflammatory factors, we adopted an ex vivo activation system using bone marrow derived macrophages.¹⁵⁹ Interestingly, there was no detectable change in expression of activation-related cell surface markers in response to either classical or alternative stimuli on macrophages derived from either miR-150KO or wild type bone marrow cells (Figure 2.9).

Interestingly, white adipose tissues from obese mice had a significantly higher portion of B lymphocytes that accounted for more than 30% of the total stromal cell population (Figure 2.8B). Although the percentages of total adipose tissue B cells were comparable in HFD-miR-150KO and HFD-WT mice, miR-150 ablation resulted in significantly more B1a cells (CD19+CD5+CD43+, $P=0.0006$) in the visceral fat tissue (average of 17.5%, Figure 2.8D) compared to controls (average of 8.6%, Figure 2.8D). Ablation of miR-150 in mice specifically enhances B1 cell formation,¹⁵⁶ a major B cell subtype in visceral adipose tissues. Thus, we hypothesized that exacerbated obesity-associated adipose tissue inflammation and insulin resistance due to miR-150 deficiency are, at least partially, attributable to alterations in the proportion of B lymphocyte subtypes.

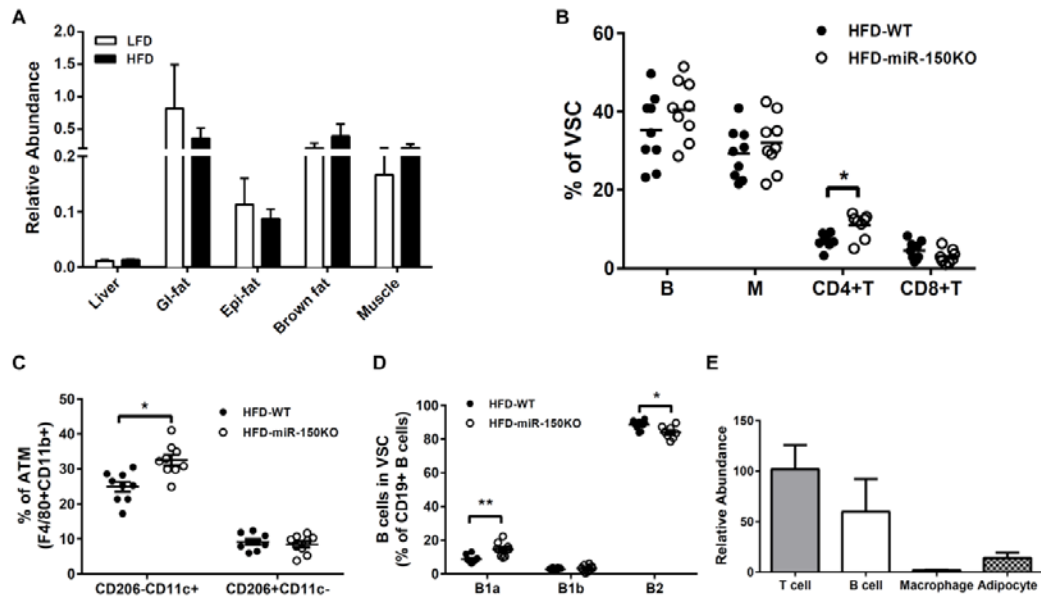


Figure 2.8 miR-150 affects the activation of immune cells in adipose tissues of mice fed a high fat diet (HFD). A, Similar expression level of miR-150 was detected in key metabolic tissues between HFD-fed and low fat diet (LFD)-fed mice (n=3). GI-fat, gastrointestinal fat. Epi-fat, epididymal fat. muscle, skeletal muscle. B, After HFD feeding, miR-150 knockout (miR-150KO) mice had greater proportion of CD4+ T cells in visceral adipose tissue (VAT) but similar population of B cells (B, CD19+), macrophages (M, F4/80+CD11b+), and CD8+ T cell (CD8+ T) compared to HFD-fed WT mice. C, There was greater proportion of M1 Adipose tissue macrophage (ATM) in vascular stromal cells (VSC) of VAT of HFD-fed miR-150KO mice, compared to HFD-fed WT mice. M1, F4/80+CD11b+CD206-CD11c+; M2, F4/80+CD11b+CD206+CD11c-. D, miR-150KO mice fed a HFD had greater population of B1 and decreased proportion of B2 cells in VSC, compared to HFD-fed WT mice. B1a, CD19+CD5+CD43+; B1b, CD19+CD5+CD43-; B2, other CD19+ B cells. E, Adipose tissue resident CD3+ T cells and B220+ B cells showed high abundance of miR-150, while F4/80+ macrophages and adipocytes had low level of this microRNA (n=3). Data are presented as mean \pm SEM. * P <0.05, ** P <0.001.

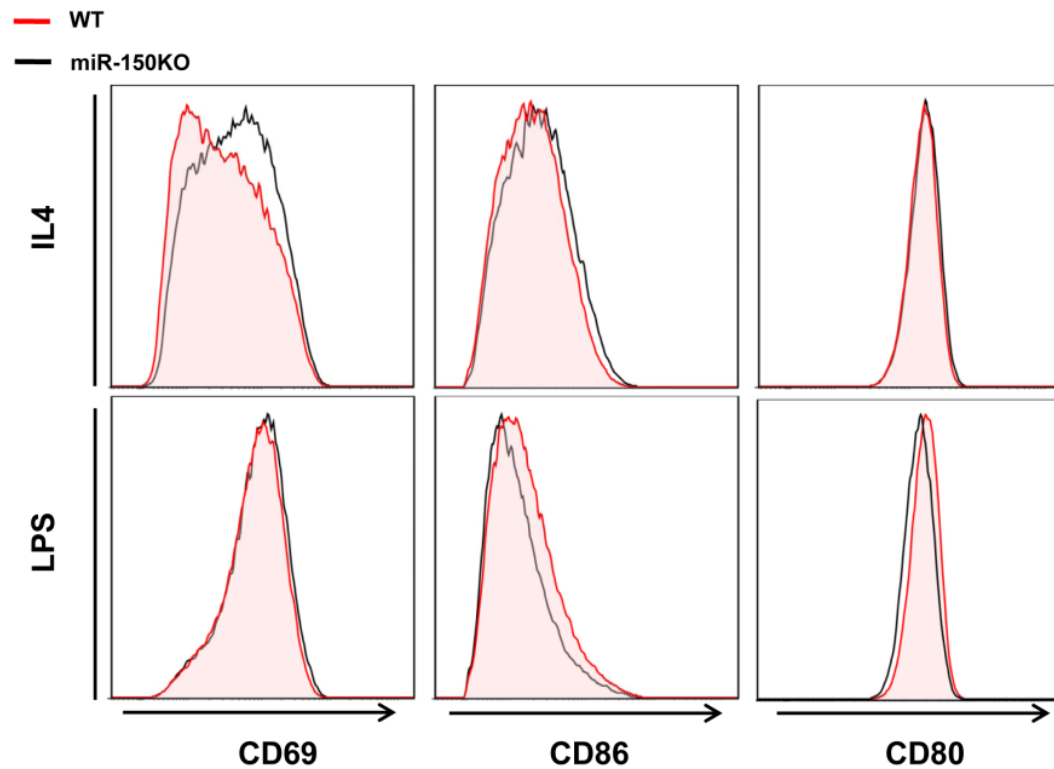


Figure 2.9 Effects of miR-150 on macrophage activation. After stimulation with either interleukin 4 (IL4) (20 ng/mL) or lipopolysaccharide (LPS; 100 ng/mL), the activation of bone marrow-derived macrophages was measured by flow cytometry with antibodies against cell surface markers CD69, CD86, and CD80. The results indicate that miR-150 did not affect the activation of macrophages. Data are presented as mean \pm SEM (n=3).

miR-150 Is an important regulator for adipose tissue B cell function

To determine the regulatory role of miR-150 in controlling B cell function, we examined its expression pattern during B cell activation. Naïve B cells isolated from spleens of 6 to 8 week-old wild type mice were stimulated using both T-cell independent (LPS) and T-cell dependent (CD4 & CD40) methodologies.^{163,164} Shortly after stimulation with LPS, there was a significant increase in expression of miR-150 in B cells that peaked during the first 24 hours and then decreased to levels comparable to those in naïve B cells after 48 hours (Figure 2.10A). Interestingly, miR-150 null B cells displayed an enhanced activation response to stimulation as evidenced by elevated activation of B cell receptor (BCR) signaling pathways and immunoglobulin production (Figure 2.10B-D).¹⁶⁵ This observation partially explained the elevated levels of plasma IgG and IgA in HFD-miR-150KO compared to HFD-WT mice (Figure 2.10E). Taken together, these results suggest that enhanced adipose tissue inflammation and exacerbated insulin resistance in obese miR-150KO mice is attributed to altered B cell functions.

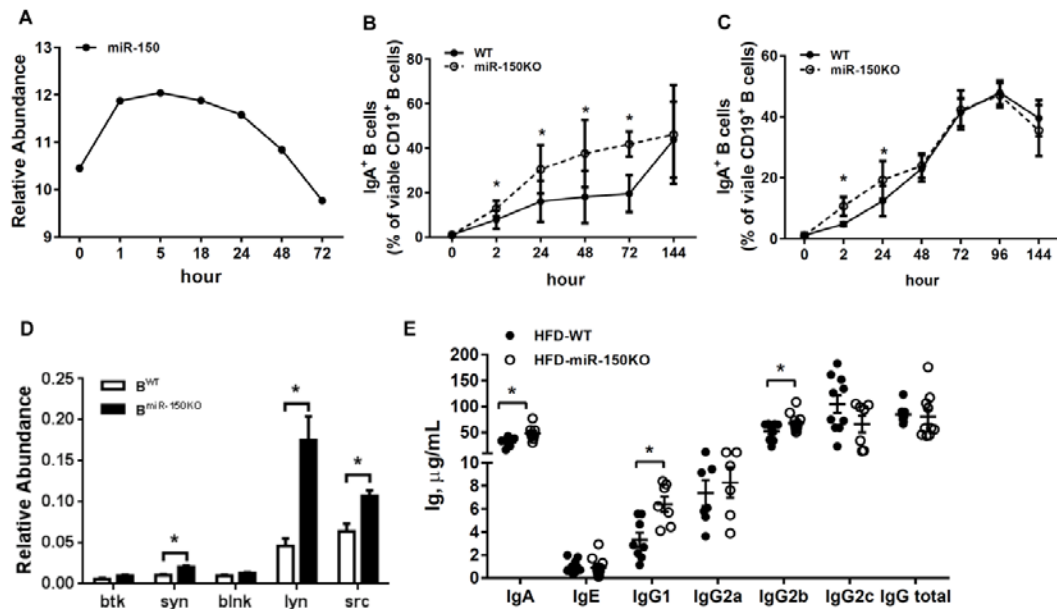


Figure 2.10 miR-150 is an important regulator of B cell function in adipose tissue. A, The expression pattern of miR-150 in B cells upon activation (n=3). B and C, miR-150 knockout (miR-150KO) B cells exhibited greater production of IgA than that of wild type (WT) B cells after stimulation with lipopolysaccharide (LPS, 10 μ g/mL) or interleukin 4 (IL4, 10 ng/mL)/CD40 (10 μ g/mL, n=9). D, miR-150KO B cells (B^{miR-150KO}) showed greater activation of B cell receptor (BCR) signaling pathway compared to WT B cells (B^{WT}) upon stimulation with LPS (n=3). E, The concentration of immunoglobulins (Ig) in plasma of WT and miR-150KO mice on a high fat diet (HFD). Data are presented as mean \pm SEM. * P <0.05.

miR-150 deficiency-dependent insulin resistance is mediated by adipose tissue B cells

To confirm the potential role of B cells in regulating obesity-associated insulin resistance, we utilized the adoptive transplantation model. B cells were isolated from HFD-miR-150KO and HFD-WT mice and injected into B^{null} mice that harbor mutated immunoglobulin μ heavy-chain, resulting in an impaired capacity for production of mature B cells (Figure 2.11).¹⁵⁸ Upon HFD feeding, B^{null} mice had significantly less insulin resistance than HFD-WT mice, which is higher in glucose intolerance compared to wild type or B^{null} mice fed the regular chow diet (LFD-WT, LFD-B^{null}, Figure 2.12A). We isolated B220+ B cells from spleens of HFD-miR-150KO (HFD-B^{null}-B^{miR-150KO}) and HFD-WT mice (HFD-B^{null}-B^{WT}), and intraperitoneally injected a total of 1×10^7 cells into B^{null} mice and maintained recipient animals on a HFD. Body weight gain, food intake and adiposity were not affected after two weeks of feeding the HFD with respect to either HFD-B^{null}-B^{miR-150KO} or HFD-B^{null}-B^{WT} mice (Figure 2.13). Examination of B cells in the visceral white adipose tissue confirmed successful engraftment (Figure 2.12B). Importantly, recipient B^{null} mice with adoptive transplants of wild type B cells displayed increased insulin resistance compared to mock transplant with PBS, which was further enhanced in recipients transplanted with miR-150KO B cells from HFD fed mice (Figure 2.12C). Accompanied by impaired glucose tolerance and insulin resistance, the abundance of total IgG and IgA in plasma was also greater in HFD-B^{null} mice engrafted with miR-150KO B cells

than WT-B cells (Figure 2.12D). These results confirm that miR-150 deficiency-induced insulin resistance is significantly influenced by B lymphocytes.

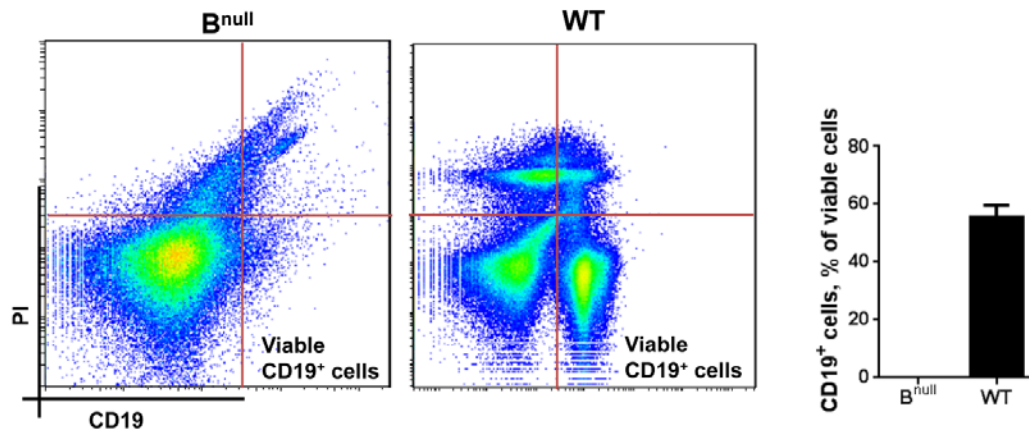


Figure 2.11 The absence of mature B cells in B^{null} mice. Viable CD19⁺ B cells in the spleen of C57BL/6 immunoglobulin μ heavy-chain knockout (B^{null}) mice and wild type (WT) mice were analyzed by flow cytometry with antibodies against CD19. The result indicates the absence of viable CD19⁺ B cells in B^{null} mice. Data are presented as mean \pm SEM (n=6).

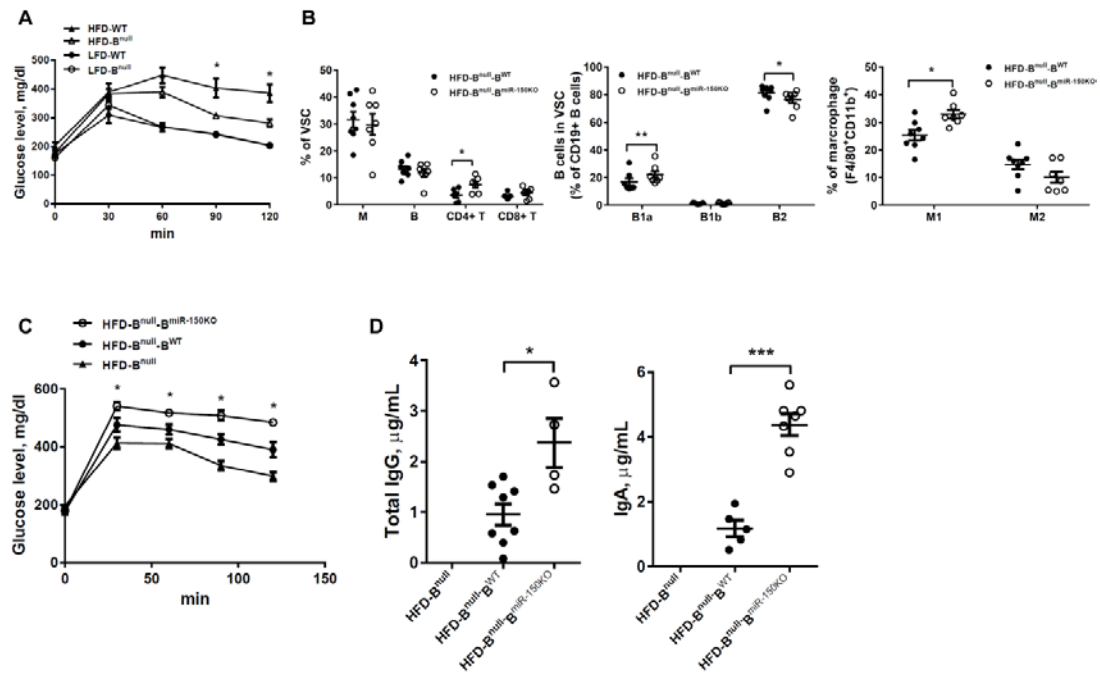


Figure 2.12 miR-150 deficiency-dependent insulin resistance is mediated by adipose tissue B cells. A, Glucose tolerance test analysis indicated that B^{null} mice fed a high fat diet (HFD-B^{null}) showed attenuated glucose intolerance compared to wild type (WT) mice on a HFD (HFD-WT; n=5-8). LFD-B^{null}, B^{null} mice was fed a low fat diet (LFD). B, The population of macrophages (M, F4/80+CD11b+), B cells (B, CD19+), CD4+ T cells (CD4+ T) and CD8+ T cells (CD8+ T), B cell subtypes, and macrophage subtypes in vascular stromal cells (VSC) of HFD-B^{null} mice at 2-weeks after injection of B cells. C, Glucose tolerance test analysis indicated that HFD-B^{null} mice with injection of miR-150KO B cells (HFD-B^{null}-B^{miR-150KO}) had greater level of glucose intolerance compared to HFD-B^{null} mice injected with WT B cells (HFD-B^{null}-B^{WT}, n=7-8). D, HFD-B^{null}-B^{miR-150KO} mice had greater levels of plasma total IgG and IgA compared to HFD-B^{null}-B^{WT} mice. Data are presented as mean \pm SEM. * P <0.05, ** P <0.001, *** P <0.0001.

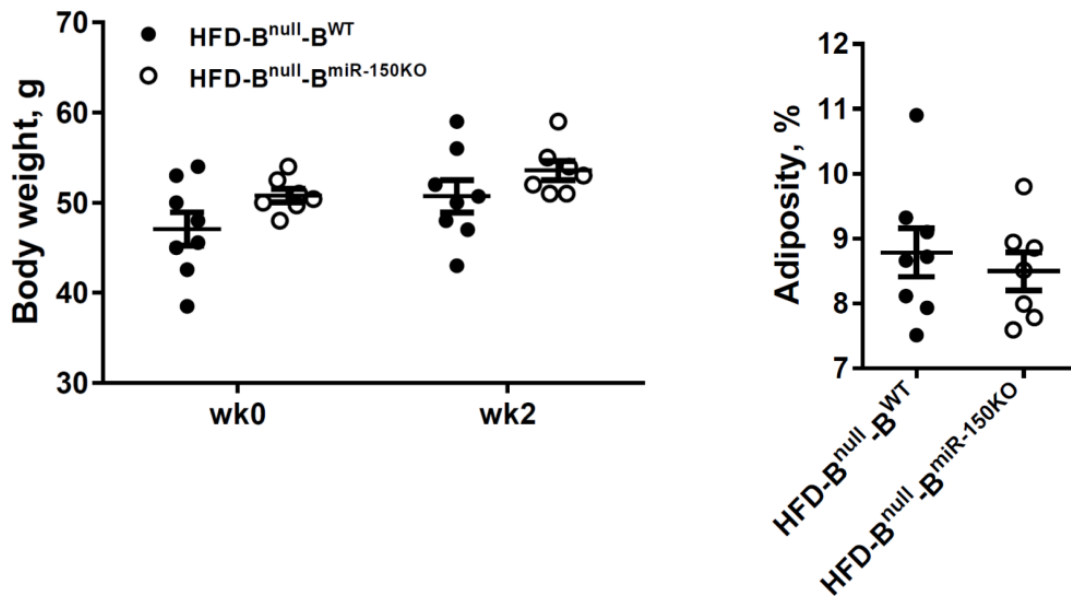


Figure 2.13 Body weight and adiposity of B^{null} mice fed a high fat diet (HFD) after B cell transplantation. The body weight and adiposity of HFD B^{null} mice were measured after injection with either wild type (WT) B cells from HFD-WT (HFD-B^{null}-B^{WT}) or miR-150KO B cells isolated from HFD-miR-150KO mice (HFD-B^{null}-B^{miR-150KO}). The results indicated that the HFD B^{null} mice with transplantation of WT or miR-150KO B cells had similar the body weight and adiposity. Data are presented as mean \pm SEM.

miR-150 regulates obesity-associated insulin resistance and tissue inflammation by controlling B cell interactions with other immune cell components in the adipose tissue niche

Results of previous studies suggest that B cells contribute to obesity-associated insulin resistance through immunoglobulin production.¹¹⁰ In this study, higher levels of immunoglobulins were also observed in both HFD-miR-150KO and HFD-B^{null}-B^{miR150KO} mice. To determine if insulin resistance observed in this model is partially attributable to alterations in immunoglobulin profiles secreted from B cells due to miR-150 depletion, we enriched plasma immunoglobulin and injected it into HFD-B^{null} mice, which had impaired antibody production due to deficient B cell formation. Surprisingly, there was no a detectable difference in insulin resistance in HFD-B^{null} mice that received immunoglobulin from either HFD-miR-150KO or HFD-WT control mice (Figure 2.14). These results suggest that the effects of miR-150 on obesity-induced insulin resistance are not related to production of pathogenic IgG antibodies.

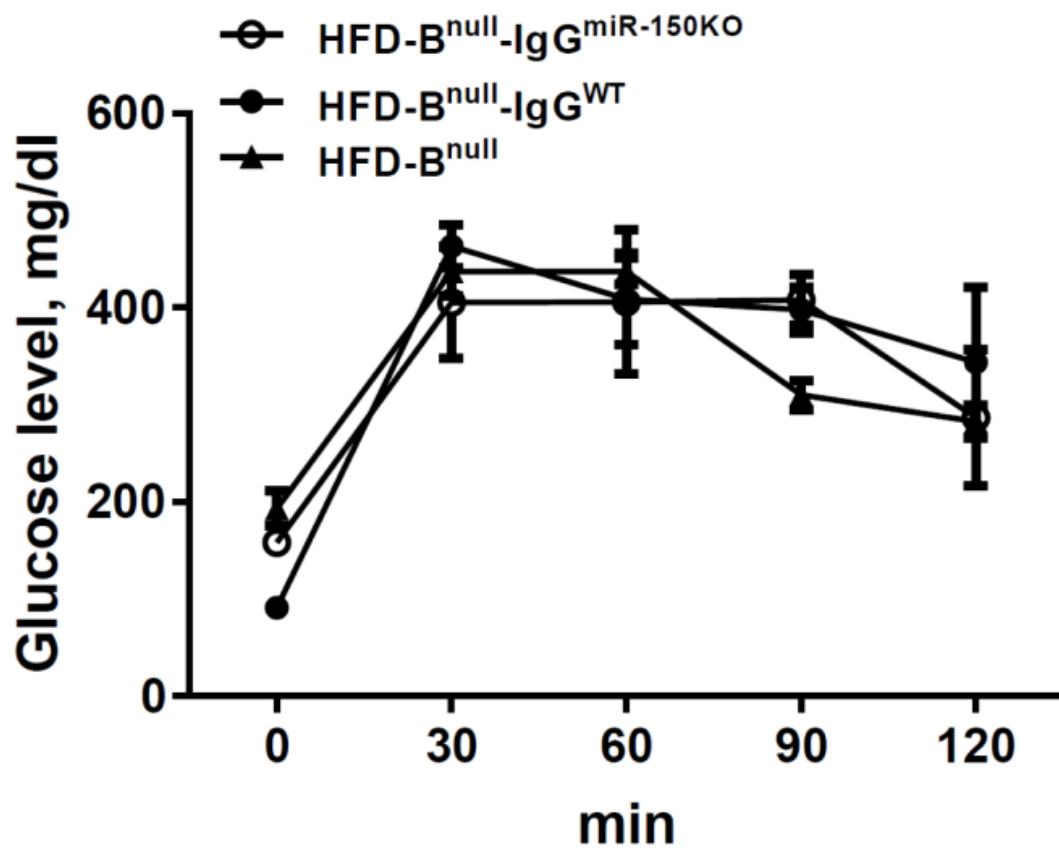


Figure 2.14 Glucose tolerance test for B^{null} mice on a high fat diet (HFD) after immunoglobulin (Ig) G injection. B^{null} mice on a HFD were injected with IgG purified from plasma of miR-150KO mice (HFD-B^{null}-IgG^{miR-150KO}) or WT mice (HFD-B^{null}-IgG^{WT}) fed a HFD. A glucose tolerance test was performed 1 week after injection of IgG. The result indicated that the effects of miR-150 on obesity-induced insulin resistance are not related to production of pathogenic IgG antibodies. Data are presented as mean \pm SEM (n=6).

In addition to immunoglobulin production, B cells play important roles in immune responses through interacting with other cell types, such as T cells and macrophages.^{166,167} Therefore, we examined whether miR-150 deficient B cells could alter B cell interactions with other major populations of immune cells in adipose tissue. Indeed, we observed enhanced activation patterns of both T cells and macrophages; the two major cell types in adipose tissue stroma. Wild type CD4⁺ and CD8⁺ T cells displayed a higher response to CD3-CD28 activation when co-cultured with activated miR-150KO B cells compared to wild type B cells, resulting in higher levels of activation-related surface markers and production of IFN γ and IL2 (Figure 2.15A and B). Although miR-150 depletion in macrophages did not alter their activation (Figure 2.9), proinflammatory M1 macrophages co-cultured with miR-150KO B cells displayed a significantly enhanced proinflammatory activation profile, including increased expression of activation-related surface markers CD69 and CD80 and production of TNF α (Figure 2.15C and D). Taken together, these results explain the overall increase in adipose tissue inflammation in obese miR-150KO mice, and suggest that miR-150 modulates adipose tissue inflammation induced by obesity by controlling B cell functions and their interactions with macrophages and T cells.

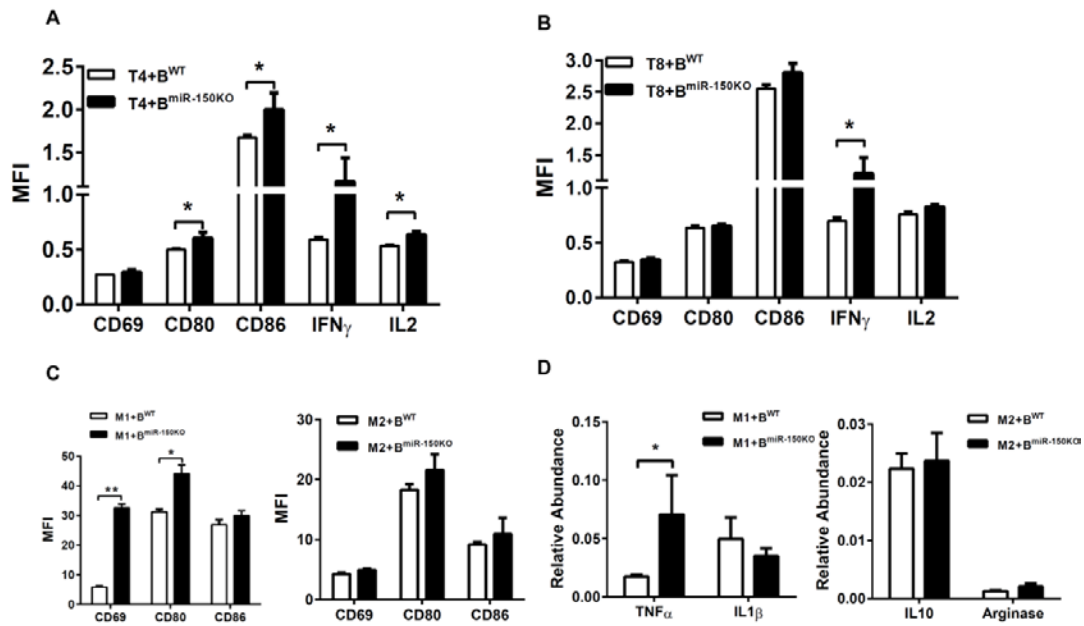


Figure 2.15 miR-150 regulates obesity-associated insulin resistance and tissue inflammation by controlling B cell interactions with other immune cells in adipose tissue. A and B, The activation of wild type (WT) CD4+ T cells (T4) or CD8+ T cells (T8) after co-culture with either WT B cells (B^{WT}) or miR-150knockout (miR-150KO) B cells (B^{miR-150KO}, n=3). C, The expression of macrophage activation-related surface markers after co-culture with B cells (n=3). D, The expression of macrophage activation-related genes after co-culture with B cells (n=3). Data are presented as mean ± SEM. **P*<0.05, ***P*<0.001.

Several genes targeted by miR-150 mediate its regulation on B cell functions

MicroRNAs exert their biological functions by either blocking translation and/or inducing degradation of the target mRNAs by base-pairing to the recognition sites.^{135,136} Previous studies have reported that *Myb* is an important target gene of miR-150 during B cell formation and activation.^{156,168-172} Utilizing prediction algorithms, TargetScan Mouse 6.2¹⁷³ and PicTar¹⁷⁴, we identified several additional genes bearing miR-150 target sites. Using a luciferase reporter assay, we confirmed that *Elk1* and *Etf1* are *bona fide* miR-150 target genes as evidenced by suppression in their luciferase activities in the presence of miR-150 (Figure 2.16A). In contrast, mutation of the miR-150 binding site in the 3' untranslated regions (UTR) of *Elk1* and *Etf1* prevented inhibition of luciferase activity by miR-150 (Figure 2.16A). Expression of miR-150 target genes *Elk1*, *Etf1*, and *Myb* was also greater in miR-150KO B cells during *in vitro* activation (Figure 2.16B). To further evaluate the effects of target genes on B cell functions, we knocked down expression of these genes in isolated B cells with shRNA constructs. The knockdown efficacy was evaluated in transfected cells by quantitative PCR analysis (Figure 2.16C and D). Intriguingly, knockdown of *Myb*, *Etf1*, *Elk1*, or all three target genes significantly decreased activation of B cells via BCR signaling pathways and production of IgA, as compared to miR-150KO B cells with an empty vector (Figure 2.16E and F). In addition, there were similar effects on B cell activation in response to knockdown of *Myb*, *Etf1*,

or *Elk1*, or all three target genes in miR-150KO B cells (Figure 2.17). These results demonstrated that *Myb*, *Elk1* and *Etf1* are bona fide targets of miR-150, which play critical roles in mediating miR-150 effects on B cell functions.

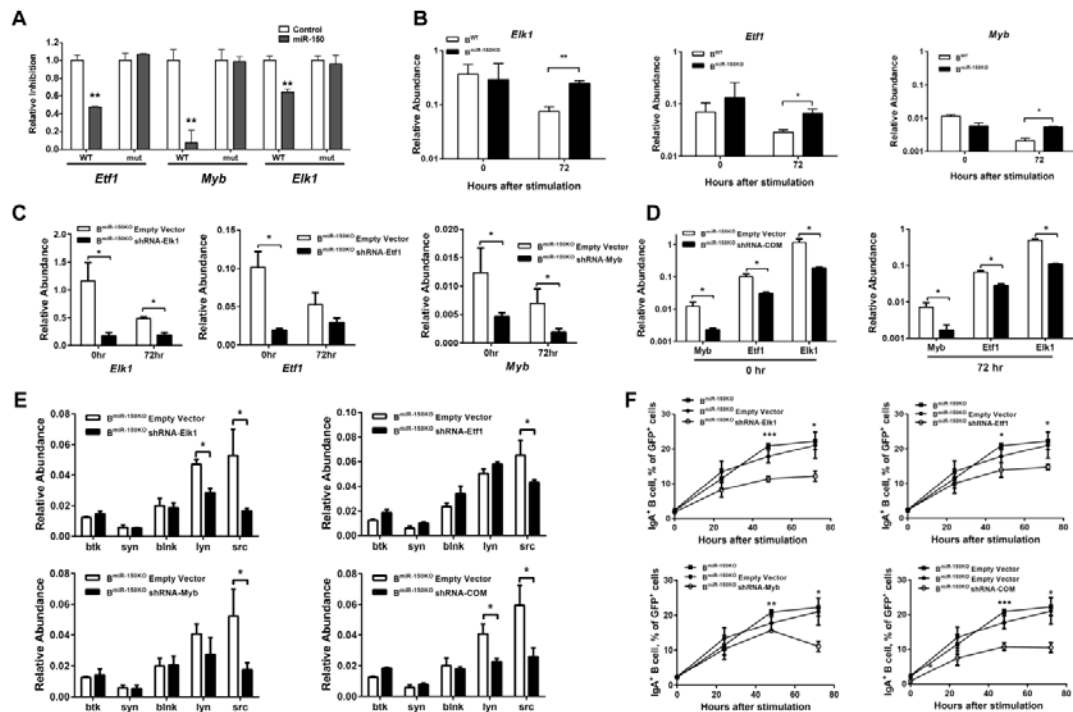


Figure 2.16 miR-150 controls B cell functions through multiple target genes. A, Reporter constructs containing a 3' untranslated region (UTR) with wild type (WT) or mutated miR-150 binding sites of target genes (mut; n=3). B, After 72-hour activation, the expression of miR-150 target genes in WT or miR-150knockout (miR-150KO) B cells (n=3). C and D, Knockdown of miR-150 target gene expression in B cells by short hairpin RNA (shRNA) assay (n=3). E, The activation of B cell receptor (BCR) signaling pathways in miR-150KO B cells with knockdown of miR-150 target genes (n=3). F, IgA production by miR-150KO B cells with knockdown of miR-150 target genes (n=6). Data are presented as mean \pm SEM. * P <0.05, ** P <0.001, *** P <0.0001. Elk1, ETS domain-containing protein. Myb, myeloblastosis oncogene. Etf1, eukaryotic translation termination factor 1.

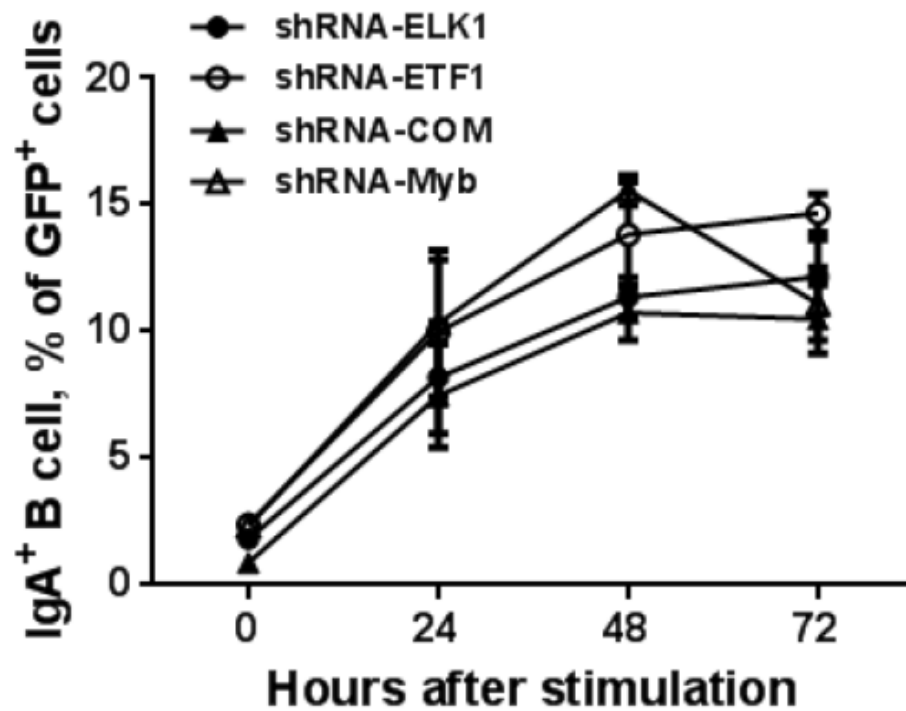


Figure 2.17 IgA production by B cells after knockdown of miR-150 target genes. IgA production by miR-150 knockout (miR-150KO) B cells after knockdown of miR-150 target genes was detected by flow cytometry with antibodies against CD19 and IgA after activation. The results indicated that knockdown of *ELK1*, *ETF1*, *Myb* or all of these three genes (COM) had similar effects on the IgA production by miR-150KO B cells. Data are presented as mean \pm SEM (n=6). Elk1, ETS domain-containing protein. Myb, myeloblastosis oncogene. Etf1, eukaryotic translation termination factor 1.

Discussion

Chronic adipose tissue inflammation is a major pathogenic phenotype associated with obesity.^{5,80,139-141} Dramatic increases in immune cell populations and enhanced inflammatory functions alter the adipose tissue phenotypes to exacerbate pre-diabetic conditions, including systemic insulin resistance and inflammation.^{44,107,110,142,143} Comparing other immune cell lineages in adipose tissue niches relevant to the diabetic condition, B cells contribute more than 20% of the stromal cell population. However, their overall impact on physiological alternations, both metabolic and inflammatory, and mechanisms underlying their action in adipose tissues of obese individuals are poorly defined. Results of this study revealed that miR-150, a hematopoietic specific microRNA, exerts regulatory effects on B cell formation and function, and it is a novel regulatory factor for obesity associated adipose tissue inflammation and insulin resistance.

Previous research, including ours, identified several hematopoietic specific microRNAs including miR-150, which is a critical regulator for B cell formation.^{155,156} Ectopic expression of miR-150 in all tissues or in hematopoietic stem cells, significantly and specifically impairs development of B lineage cells by blocking the transition from pro-B to pre-B stages.¹⁵⁵ Interestingly, deletion of miR-150 resulted in a milder phenotype overall with elevated B1 cell production, but non-detectable changes in other hematopoietic lineages or non-hematopoietic tissues.¹⁵⁶ Although B1 cells account for less than 5% of the total

B cells in circulation,^{152,157} they are one of the major immune cell types in peritoneal fluid and tissues.^{147,149,152} Thus, the miR-150 knockout mouse is a unique animal model to investigate the impact of B cells, including B1 cells, on development of chronic inflammation and insulin resistance in response to the stress of obesity. Indeed, we observed exacerbated pre-diabetic conditions, including elevated levels of insulin and cytokines, in miR-150KO mice compared to wild-type controls fed a HFD. Further, we now confirm that B1 cells are a major immune cell lineage in adipose tissue, accounting for 3% in lean mice and significantly increased to 8% in obese mice (Figure 2.8D). In addition, mice with miR-150 deficiency displayed even a higher proportion of B cell infiltration into adipose tissue represented by 77% B2, 16% B1a, and 7% B1b cells (Figure 2.8d). Further, deletion of miR-150 in B cells significantly altered B cell activation in response to both T cell-dependent and T cell-independent stimuli with respect to increasing activation of BCR signaling pathways and immunoglobulin production (Figure 2.10).

Given that previous studies suggest a minimal impact of miR-150 deficiency on T cell formation and activation,¹⁵⁶ we examined the potential impact of the miR-150 mutation in macrophages, the major immune cell population in adipose tissue niches. Interestingly, loss of miR-150 in macrophages did not alter macrophage polarized activation states with respect to activation related surface markers.

Our adoptive transplantation model of B^{null} mice allowed the determination that transfer of B cells from HFD-miR150 or HFD-WT partially recapitulates insulin resistance characteristic of miR-150 mutant mice (Figure 2.12C). Winer et al. suggested that immunoglobulin production by B cells contributes to insulin resistance;¹¹⁰ however, we failed to observe such an effect in response to purified immunoglobulin (primarily IgG with a small portion of IgA and others) from HFD-miR-150KO or HFD-WT mice. We observed that B cells lacking miR-150 displayed a slight increase in immunoglobulin production, which could partially contribute to higher circulating levels of IgG and IgA in plasma of mice fed a HFD. However, the specific contribution of immunoglobulin production by B cells, especially adipose tissue B cells, to the development of insulin resistance needs to be investigated further. Taken together, our results suggest that adipose tissue B cells, including the B1 subtype, are primary contributors to enhanced adipose tissue inflammation and systemic insulin resistance in obese miR-150KO mice.

Although loss of miR-150 did not significantly alter macrophage and T cell activation states under obesity stress directly (Figure 2.9),¹⁵⁶ an enhanced overall proinflammatory profile was detected (Figure 2.6). We confirmed that miR-150KO B cells can enhance both T cell and macrophage activation profiles (Figure 2.15) which can impair insulin tolerance in adipose tissue with transplanted B cells lacking miR-150 (Figure 2.12C). In addition, enhanced T cell and macrophage infiltration into adipose tissue in the adoptive transplantation

model suggests that adipose tissue B cells are a potent source of cytokines for recruitment of immune cells to adipose tissue under the stress of obesity. Together with increased T cells and macrophages, adipose tissue B cells exacerbate obesity induced chronic inflammatory features in adipose tissue and subsequent systemic insulin resistance (Figure 2.18).

Our understanding of the contributions of B cells to adipose tissue functions, especially under the stress of obesity, is in its infancy. In this study, we demonstrate that miR-150, a B cell specific regulatory microRNA, is a critical modulator of the pathogenesis of chronic tissue inflammation and insulin resistance associated with obesity, which are major contributors to metabolic syndrome and development of type 2 diabetes and other cardiovascular complications. Simultaneous targeting of multiple targets of miR-150 can exert a potent impact by shifting the signaling network governing adipose tissue B cell function, which can, in turn, change the adipose tissue niche and its function. Further investigations regarding the mechanisms of action of miR-150 and B cells will provide important information toward development of therapeutic strategies to alter microRNAs and modulate B cells to treat obesity-associated complications.

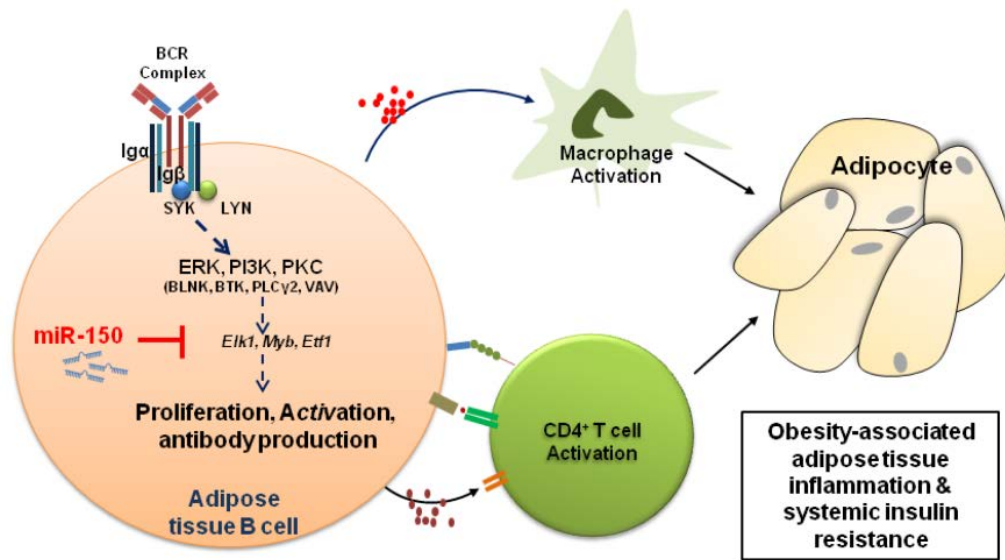


Figure 2.18 Schematic model of miR-150-mediated molecular network in B cells. miR-150 directly suppresses the expression of target genes, *Elk1*, *Myb*, *Etf1*, which are critical mediators of the activation of B cell receptor (BCR) signaling cascade. This miR-150-mediated B cell function is also critical for the activation of CD4⁺ T cells and macrophages residing in adipose tissues, eventually exerting profound impacts on the function of adipocytes. Elk1, ETS domain-containing protein. Myb, myeloblastosis oncogene. Etf1, eukaryotic translation termination factor 1. SYK, spleen tyrosine kinase. LYN, tyrosine-protein kinase. ERK, extracellular regulated MAP kinase. PI3K, phosphoinositide 3-kinase. PKC, protein kinase C. BLNK, B-cell linker. BTK, Bruton's tyrosine kinase. PLCγ2, phospholipase C, gamma 2. VAV, guanine nucleotide exchange factor.

CHAPTER III

REGULATORY AXIS OF PPAR γ -MIR-223 IN CONTROLLING ACTIVATION OF ADIPOSE TISSUE MACROPHAGES

Adipose tissue macrophages (ATMs) are a critical cellular component for adipose tissue function. We recently reported that miR-223 is a potent regulator for activation of ATMs that favors an anti-inflammatory M2 phenotype. However, it is unclear if the regulation of miR-223 and subsequent interactions are consistent with known regulatory signaling pathways. I sought to determine the regulatory mechanism controlling miR-223 expression during ATM polarization, with a focus on PPAR γ -dependent M2 activation. By employing both *in vivo* and *in vitro* models, I demonstrated that miR-223 is a critical mediator for PPAR γ action in controlling M2 ATM activation, and subsequent beneficial effects to mitigate tissue inflammation and insulin resistance. I observed impaired PPAR γ functions in miR-223KO mice with respect to suppression of inflammation and insulin sensitivity. I further demonstrated that miR-223 is a key mediator of PPAR γ -regulated macrophage activation. Disruption of miR-223 in macrophages impaired PPAR γ -dependent M2 activation, whereas over-expression of miR-223 further enhanced M2 activation in response to PPAR γ activation. Genomic screening of the miR-223 promoter identified three PPAR γ -regulatory elements followed by confirmation with chromatin immunoprecipitation analysis and a luciferase reporter assay. My results revealed that two new miR-223 target

genes, *Rasa1* and *Nfat5*, are crucial regulators of M2 activation via the PPAR γ -miR-223 axis. This study provides the first evidence supporting a crucial role for the PPAR γ -miR-223 axis in controlling activation of ATMs under the stress of obesity. In addition, identification of novel target genes in this context provided new genes for development of therapeutics agent to mitigate obesity related diseases.

Introduction

Adipose tissue resident macrophages (ATMs) exhibit heterogeneous phenotypes in response to various microenvironmental signals, ranging from proinflammatory M1 activation to anti-inflammatory M2 responses.^{175,176} Upon stimulation by T helper type 1 (Th1) cytokines, lipopolysaccharide (LPS) or free fatty acids, ATMs exhibit proinflammatory M1 responses,^{13,60} whereas Th2 cytokines, including interleukin 4 (IL4) and IL13, induce activation of anti-inflammatory M2 ATMs.^{66,67} The polarized activation status of ATMs is critical for maintaining adipose tissue homeostasis. In the context of obesity, ATMs preferentially undergo proinflammatory M1 activation, which propagates the chronic inflammatory status and insulin resistance.^{43,177,178} However, the regulatory mechanisms underlying activation of ATMs has not been fully elucidated.

Peroxisome proliferator-activated receptor γ (PPAR γ), a nuclear transcription factor, exerts profound functions that control polarized activation of

ATMs. Activation of the PPAR γ signaling pathway in ATMs suppresses the proinflammatory M1 phenotype by blocking activation of the nuclear factor κ B (NF κ B) cell signaling pathway.⁶⁸⁻⁷⁰ Activated PPAR γ also induces expression of M2 response-related genes which involve fatty acid metabolism and mitochondrial activities.^{66,179-182} Furthermore, the PPAR γ -mediated activation pattern of ATMs is sufficient to regulate adipose tissue functions and insulin resistance under the stress of obesity.^{66,183,184} Deletion of PPAR γ in ATMs results in increased M1 activation and blunted M2 responses, which in turn exacerbates adipose tissue inflammation and reduces insulin sensitivity.⁶⁶

Our recent study identified microRNA-223 (miR-223) as an important regulator of activation of ATMs favoring the anti-inflammatory M2 phenotype.⁹³ ATMs with miR-223 deletion elicited an enhanced proinflammatory M1 phenotype, but less anti-inflammatory M2 responses under the stress of obesity. This resembles the phenotypes derived from knockout of PPAR γ in macrophages. Furthermore, the exacerbated adipose tissue inflammation and systemic insulin resistance associated with type 2 diabetes were driven by alterations in patterns of activation of ATMs in miR-223KO mice. These results demonstrate that miR-223 exerts protective effects on the pathogenesis of obesity-associated metabolic disorders through controlling ATM activation. However, the regulatory mechanism for miR-223 expression during ATMs polarized activation, and interactions of miR-223 with the regulatory signaling

pathways governed by key regulators such as PPAR γ in responding to stimuli in the obese tissue niche are not established.

In this study, we demonstrated the miR-223 is required for PPAR γ action and, in turn, ATM activation and subsequent insulin sensitivity under the stress of obesity. In addition, we identified three PPAR γ regulatory elements located in the promoter region of the miR-223 gene, suggesting that a PPAR γ -miR-223 regulatory axis modulates ATM polarization, hence controlling immune homeostasis of the adipose tissue niche.

Materials and Methods

Animal experiments

The generation of miR-223 deficient (miR-223KO) mice has been described previously.¹⁸⁵ Wild-type (WT) C57BL/6J mice were used as controls. All mice were maintained on a 12/12-hour light-dark cycle. Male mice 5 to 6 weeks of age were fed *ad libitum*. Mice were fed a high-fat diet (HFD; Cat. No. D12492, Research Diets, Inc; Table A-1). After 12-week feeding, either pioglitazone (10 mg/kg/day; stimulating PPAR γ activation; ACTOS[®])¹⁸⁶ or phosphate buffered saline (PBS) was delivered into HFD-fed mice via intragastric injection for 4 weeks. After the feeding regimen, mice were subjected to phenotypic characterization and metabolic assays, including measurement of metabolic parameters in plasma, as well as insulin and glucose tolerance tests.

All study protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Texas A&M University.

Isolation of stromal cells, mature adipocytes, and macrophages from visceral adipose tissues (VATs)

The visceral adipose tissues were mechanically dissected and then digested with collagenase II (2 mg/mL; Cat. No. 17101-015, Invitrogen; Table A-3) for 30 min at 37°C. After lysing red blood cells and passing the cells through a 200-µm cell strainer, visceral fat stromal cells (VSC) and mature adipocytes were separated by centrifugation at 1000 x g for 5 min. To purify ATMs, VSC were incubated with biotin-labeled antibody against F4/80 (Cat. No. 13-4801-85, eBioscience) for identification of macrophages. Magnetic beads conjugated with streptavidin (Cat. No. 557812, BD Biosciences) were applied for isolation of macrophages.

Macrophage differentiation and polarization

Bone marrow-derived macrophages (BMDMs) were prepared as previously described.¹⁶⁰ Macrophage maturation was examined by flow cytometry with antibodies against cell surface markers F4/80 (Cat. No. 53-4801-82, eBioscience) and CD11b (Cat. No. 12-0112-83, eBioscience). BMDMs were stimulated with LPS (100 ng/mL) for M1 activation or IL4 (20 ng/mL) for M2 activation. To enhance activation of PPAR γ , BMDMs were treated with pioglitazone (1 µM).¹⁸⁷ BMDMs were treated with a PPAR γ antagonist, GW9662

(0.1 μ M; Cat. No. 70785, Cayman Chemical) to suppress activation of PPAR γ .¹⁸⁸

Flow cytometry analysis

The VSC, ATMs, and BMDMs were stained with fluorescence-tagged antibodies (0.125 μ g/ 10^6 cells) to detect cell lineages. Myeloid cells were detected using antibodies against cell surface antigens F4/80 (Cat. No. 11-4801-82, eBioscience), CD11b (Cat. No. 45-0112-82, eBioscience) and Gr-1 (Cat. No. 35-5931-82, eBioscience); macrophage subtypes were detected with antibodies against cell surface markers F4/80, CD11b, CD206 (Cat. No. 141706, Biolegend), CD11c (Cat. No. 12-0114-82, eBioscience). Macrophage activation was measured with antibodies against cell surface proteins CD80 (Cat. No. 17-0801-82, eBioscience), CD69 (Cat. No. 45-0691-82, eBioscience), and CD86 (Cat. No. 11-0862-82, eBioscience). Data were analyzed using Flowjo software or Accuri C6 software (BD Biosciences) or Kluza software (Beckman Coulter).

Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from VATs, ATMs, adipocytes and BMDMs using the Trizol extraction protocol according to the manufacturer's instructions (Cat. No. R2050, Zymo Research). Gene expression analysis was performed using a iScript One-Step RT-PCR kit with SYBR Green (Cat. No. 170-8893, Bio-Rad) on Bio-Rad CFX384 (Bio-Rad). The data presented correspond to the

mean of $2^{-\Delta\Delta Ct}$ from at least three independent experiments after being normalized to β -actin.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed as described previously.¹⁸⁹ Briefly, BMDMs were cross-linked for 10 min with 1% formaldehyde and quenched with 125 mM glycine (Table A-5). After nuclei were isolated by centrifugation, the pellet was resuspended in lysis buffer containing 0.1% SDS and sonicated to achieve fragment sizes of 200-600 bp by Bioruptor® Pico (diagenode). The immunoprecipitation was conducted with ChIP-grade protein G magnetic beads (Cat. No. 9006S, Cell Signaling) using an antibody against PPAR γ (Cat. No. ab41928, Abcam). IgG protein (Cat. No. ab18413, Abcam) was used as the negative control. To validate the enrichment, quantitative PCR was performed with tiled primers.

Bio-Plex protein expression assay

The concentrations of TNF α , IL1 β , and IFN γ in plasma were determined using a Bio-Plex™ Cytokine Assay (Cat. No. M60-009RDPD, Bio-Rad). Concentration of insulin in plasma was determined using the Bio-Plex Pro Mouse Diabetes Insulin set (Cat. No. 171-G7006M, Bio-Rad). The abundance of total (Cat. No. 171-V60007M, Bio-Rad) and phosphorylated (Cat. No. 171-V50011M, Bio-Rad) c-Jun N-terminal kinases (JNK) and phosphorylated P65 (Cat. No. 171-V50013M, Bio-Rad) in VAT was determined using the Bio-Plex

Cell Signaling Magnetic Assays (Bio-Rad). β -actin was used as the internal control. These Bio-Plex assays were performed using the Bio-Plex MAGPIX™ multiplex reader (Bio-Rad). Results were analyzed using Bio-Plex Data Pro™ software (Bio-Rad).

Luciferase reporter assay

The luciferase reporter assay was carried out as described previously.⁹³ To verify that PPAR γ binds to the upstream region of pre-miR-223, a 1732-bp DNA fragment (-3849 to -2117 relative to the 5' end) within the upstream region of the pre-miR-223 gene was inserted into upstream of the SV40 promoter of the pGL3 promoter vector (Promega). The luciferase activity was determined by transient transfection of the murine macrophage cell line RAW264.7 with Bright-Glo luciferase reporter system (Promega) and normalized to the internal control firefly luciferase activity. The full 3'-untranslated region (3'-UTR) sequence of these genes or at least the 250 bp flanking region of the predicted miR-223 binding site was cloned into the psiCheck2 Vector (Promega) downstream of the *Renilla* luciferase-coding region. To validate the suppressive effects of miR-223, the reporter constructs were co-transfected with miR-223 mimic oligonucleotides or negative control oligonucleotides into HEK293 cells. After 48 h co-transfection, the activities of *Renilla* luciferase were measured using the Dual-Glo luciferase reporter system (Cat. No. E2920, Promega) and normalized to the internal control firefly luciferase activity. Repressive effects of miR-223 on target genes

were plotted as the percentage repression in three biological repeats that each contained three technical repeats.

Lentiviral shRNA assay

The pLKO.1-CMV-TurboGFPTM vector (Sigma-Aldrich) with inserted short hairpin RNA (shRNA; targeting *Nfat5* and *Rasa1*) was co-transfected with compatible packaging plasmids into HEK293T cells. The lentiviral supernatants were collected 72 h after transfection and used to infect BMDMs. The empty vector was used as the control.

Data and statistical analyses

Results are expressed as means \pm SEM. Each data point derived from RT-PCR assays represents an average of two technical replicates, and data were averaged over independently replicated experiments (n=3-4 independently collected samples) and analyzed using the Student's *t* test. The overall group-effect was analyzed for significance using two-way ANOVA and the Bonferroni post-test for each factor at each individual time. Data analyses were performed using Graphpad Prism version 6.0 software. A value of $P < 0.05$ was considered statistically significant.

Results

miR-223 Is required for PPAR γ -dependent activation of adipose tissue macrophages under obesity-induced stress

As an insulin sensitizer, PPAR γ activation improves obesity-associated metabolic abnormalities.¹⁹⁰⁻¹⁹² Indeed, after administration of the PPAR γ agonist pioglitazone for 4 weeks, high fat diet-fed wild type mice (HFD-WT-pio) showed a remarkable improvement in insulin sensitivity based on results from both the glucose tolerance test and the insulin tolerance test (Figure 3.1A and B). In addition, HFD-WT-pio mice had reduced hyperglycemia and hyperinsulinemia compared to HFD-WT mice (Figure 3.1C). No significant changes were observed in body weight gain and food intake among the HFD-fed mice (Figure 3.2). The improved insulin sensitivity was accompanied with the attenuated inflammatory responses.¹⁷⁷ As expected, we observed reduced inflammatory responses in visceral fat depots of HFD-WT-pio mice, as evidenced by decreased activities of JNK and NF κ B signaling pathways and expression of inflammatory cytokines (Figure 3.1D-F). Concomitantly, we detected a significant decrease in concentrations of inflammatory cytokines including TNF α and IL1 β in plasma of HFD-WT-pio mice compared to HFD-WT mice (Figure 3.1G).

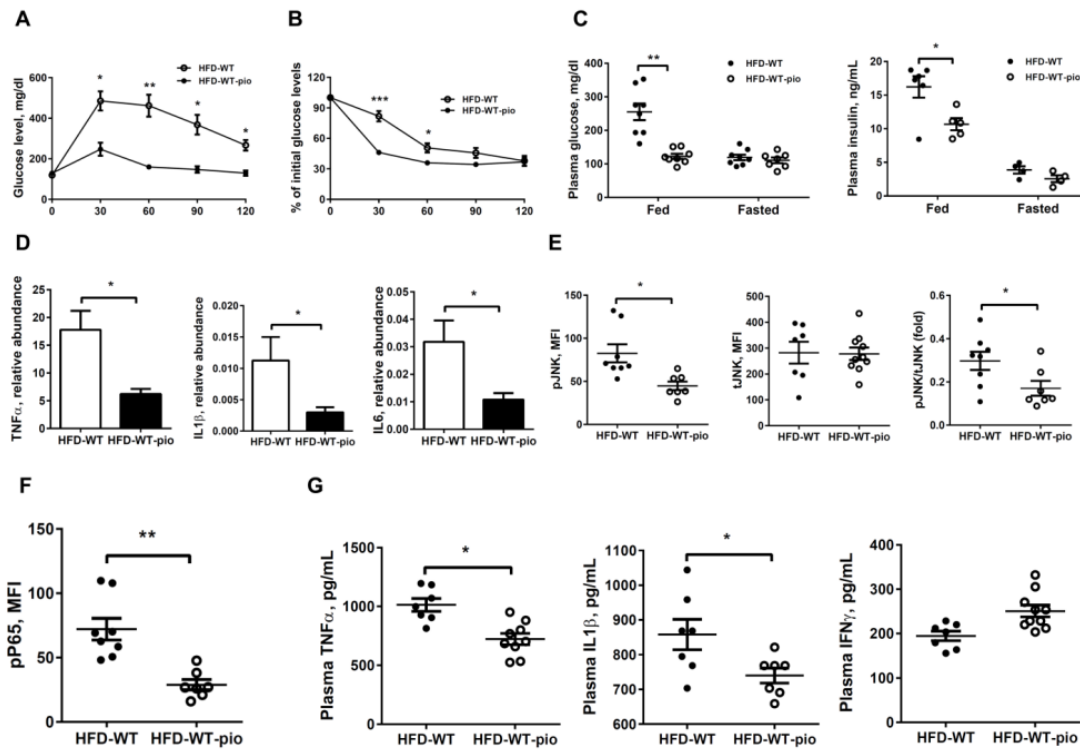


Figure 3.1 Enhanced activation of PPAR γ improves obesity-associated adipose tissue inflammation and insulin sensitivity in wild type (WT) mice and WT mice treated with pioglitazone (WT-pio) on a high fat diet (HFD). A and B, results of glucose tolerance and insulin tolerance tests (n=9-10) indicate PPAR γ promotes insulin sensitivity of obese mice. C, Concentrations of glucose and insulin were less in plasma of WT mice fed a HFD with PPAR γ agonist pioglitazone treatment (HFD-WT-pio) than that of obese without pioglitazone treatment (HFD-WT) but no difference when mice were fasted for 16 hours. D, Expression of genes for proinflammatory cytokines tumor necrosis factor- α (TNF α), interleukin (IL)1 β , and IL6 was reduced in visceral adipose tissue (VAT) of WT-pio mice fed a HFD (n=3). E and F, Activation of c-Jun N-terminal kinases (JNK) and nuclear factor- κ B (NF κ B) signaling pathways in VAT was repressed after administration of pioglitazone. MFI, medium fluorescence intensity. G, Concentrations of proinflammatory cytokines in plasma of mice fed a HFD were decreased after administration of pioglitazone. IFN γ , interferon γ . Data are presented as mean \pm SEM. * P <0.05, ** P <0.001.

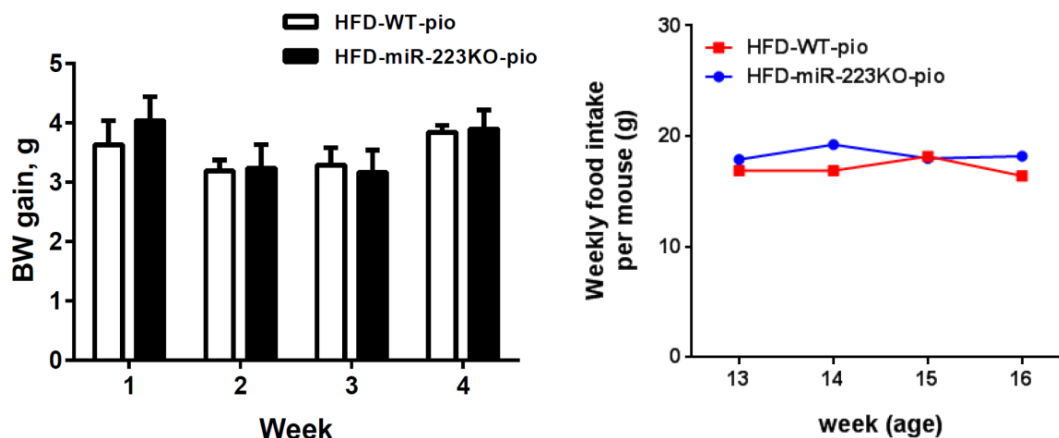


Figure 3.2 Body weight (BW) gain and food intake. After being fed a high fat diet (HFD) for 12 weeks, wild type (HFD-WT-pio) and miR-223 deficient (HFD-miR-223KO-pio) mice were given daily intragastric injection of pioglitazone (10 mg/kg/day) or phosphate buffered saline (PBS) for an additional 4 weeks. Body weight and food intake of mice were recorded every week during the feeding regimen. The results indicated that PPAR γ activation had minimal effects on weight gain and food intake of obese mice. Data are presented as mean \pm SEM (n=9-10).

In the context of obesity, enhanced activation of PPAR γ significantly suppresses inflammatory responses of ATMs and this is critical for attenuating inflammation in adipose tissue. After administration of pioglitazone for 4 weeks, we observed comparable populations of ATMs between HFD-WT and HFD-WT-pio mice, accounting for about 30% of stromal cells (Figure 3.3A). This indicates that activation of PPAR γ had minimal effects on the infiltration of macrophages into VAT of HFD-fed mice. However, PPAR γ -dependent responses included a decrease in the proportion of proinflammatory M1 ATMs and increased M2 ATMs in VAT of HFD-fed WT mice treated with pioglitazone (Figure 3.3A). Interestingly, expression of miR-223 was induced in ATMs, but not adipocytes of HFD-WT mice after treatment with pioglitazone for 4 weeks (Figure 3.3B). These results suggest that miR-223 plays a role in the PPAR γ -dependent cell signaling pathway that modulates activation of ATMs.

We further investigated the impact of miR-223 on PPAR γ action to regulate ATM activation using miR-223 deficient (miR-223KO) mice. Intriguingly, in contrast to the suppression on proinflammatory M1 responses in ATMs observed in HFD-WT-pio mice (Figure 3.3A), the activation pattern for ATMs was similar between HFD-miR-223KO mice and HFD-miR-223KO-pio mice (Figure 3.4A), suggesting that miR-223 depletion impaired the ability of PPAR γ to modulate the activation status of ATMs. We also observed that the exacerbated inflammatory responses observed in HFD-miR-223KO mice were not fully recovered by pioglitazone treatment, as judged by comparable degrees

of activation of JNK/NF κ B signaling pathways and production of proinflammatory cytokines (Figure 3.4B-E). In addition, pioglitazone treatment attenuated glucose intolerance, but not insulin resistance of HFD-miR-223KO mice (Figure 3.4F-G). Thus, these results suggest that loss of miR-223 impairs PPAR γ functions that mediate activation of ATMs and subsequent inflammatory responses and insulin resistance.

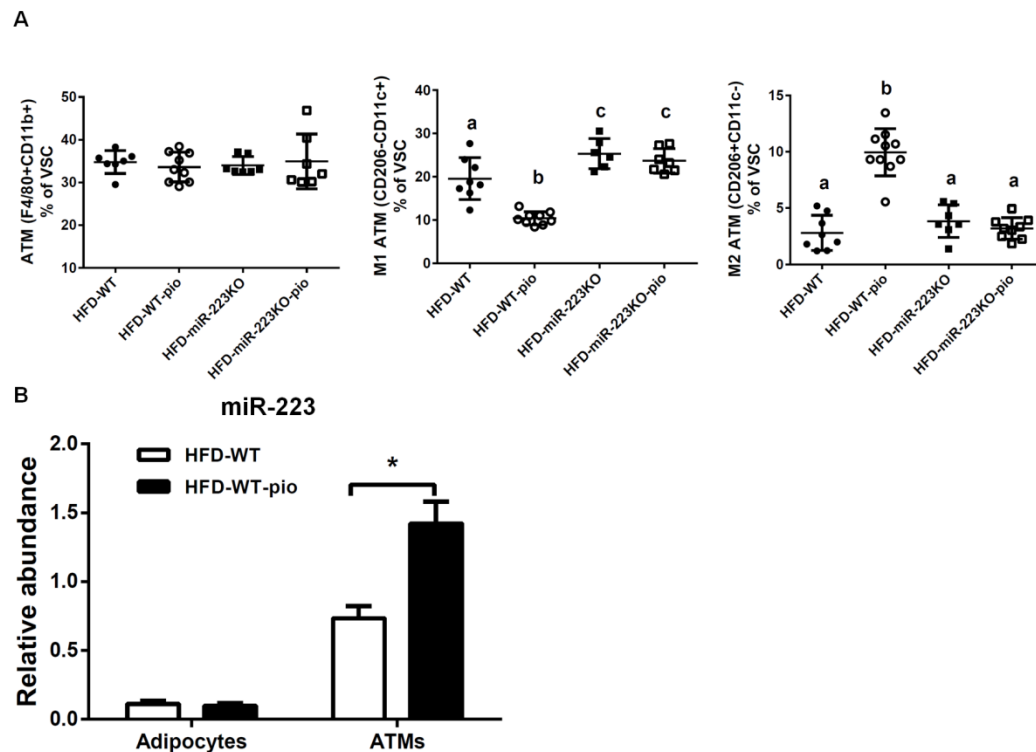


Figure 3.3 The expression of miR-223 is induced via PPAR γ -mediated activation of adipose tissue macrophages. A, The population of macrophages (F4/80+CD11b+) and their subtypes in vascular stromal cells (VSC) of visceral adipose tissue (VAT) from mice fed a high fat diet (HFD). M1, F4/80+CD11b+CD206-CD11c+; M2, F4/80+CD11b+CD206+CD11c-. B, The expression of miR-223 in adipocytes and ATMs of mice fed a HFD was induced due to treatment with pioglitazone. Data are presented as mean \pm SEM (n=3). * P <0.05.

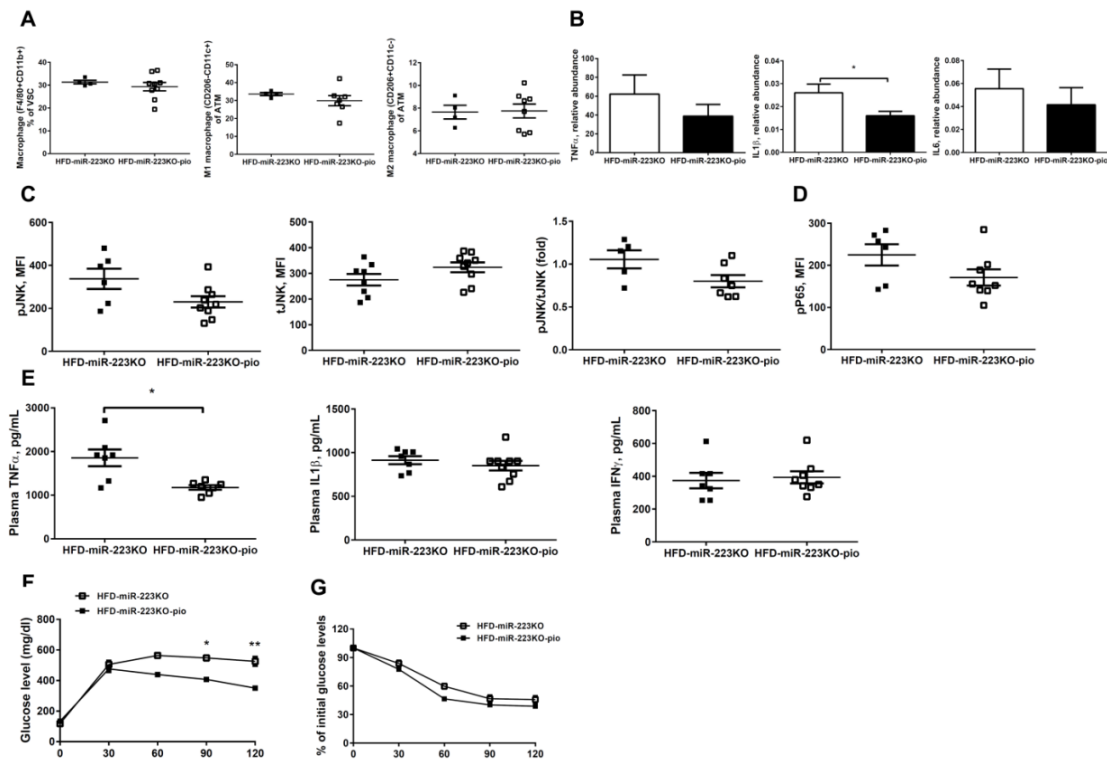


Figure 3.4 A deficiency in miR-223 impairs PPAR γ functions in modulating adipose tissue functions and macrophage activation under the stress of obesity. A, The population of macrophage and its subtypes in visceral adipose tissue (VAT) were comparable between miR-223KO mice fed a high fat diet (HFD) with or without treatment with pioglitazone (HFD-miR-223KO-pio, HFD-miR-223KO). B, Pioglitazone treatment did not fully repress the expression of genes for proinflammatory cytokines TNF α , IL1 β , and IL6 in VAT of miR-223KO mice fed a HFD (n=3). C and D, Activation of JNK and NF κ B signaling pathways in VAT were similar between HFD-miR-223KO and HFD-miR-223KO-pio mice. MFI, medium fluorescence intensity. E, Concentrations of proinflammatory cytokines in plasma of miR-223KO mice fed a HFD were decreased after administration of pioglitazone. F and G, Results of glucose tolerance test and insulin tolerance test indicated that pioglitazone treatment failed to fully recover the insulin sensitivity of miR-223KO mice fed a HFD (n=9-10). Data are presented as mean \pm SEM. * P <0.05, ** P <0.001.

The regulatory axis of PPAR γ -miR-223 controls M2 responses

Given the significant impacts of miR-223 on PPAR γ -mediated activation of ATMs, we further explored the role of miR-223 in PPAR γ signaling pathway using an *ex vivo* model with BMDMs. We observed that pioglitazone treatment further enhanced M2 responses of BMDMs induced by IL4, as evidenced by the increased expression of activation-related cell surface markers (WT-IL4+pio vs. WT-IL4; Figure 3.5A). However, BMDMs lacking miR-223 exhibited a blunted M2 response that was not fully rescued by pioglitazone treatment with respect to decreased the expression of activation-related surface marker CD69 and other key genes such as peroxisome proliferator-activated receptor gamma, coactivator 1 α (PGC1 α), IL10, and arginase 1 (Figure 3.5A and B). This recapitulated the phenotypes of HFD-miR-223KO mice treated with pioglitazone (Figure 3.4A). Conversely, over-expression of miR-223 in BMDMs enhanced M2 responses (WT-IL4 vs. miR223oe-IL4), which was similar to the improvement in expression of activation-related surface markers of WT BMDMs induced by pioglitazone treatment (miR223oe-IL4 vs. WT-IL4+pio; Figure 3.5C). Further, pioglitazone treatment increased the expression of activation-related cell surface marker CD69 in BMDMs with ectopic expression of miR-223 (miR223oe-IL4+pio vs. WT-IL4+pio; Figure 3.5C). Taken together, these results demonstrate that miR-223 acts as a critical component of the PPAR γ -dependent signaling pathway that regulates activation of M2 macrophages.

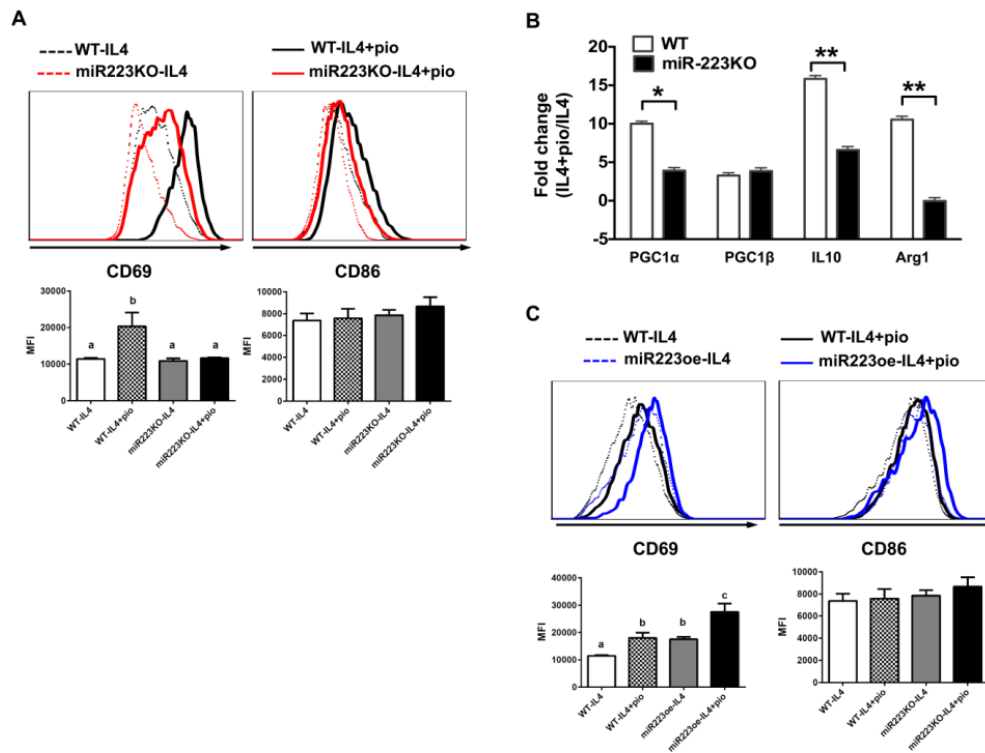


Figure 3.5 miR-223 is required for PPAR γ -dependent activation of alternative M2 macrophages. A, The expression of activation-related cell surface markers, CD69 and CD86, of bone marrow derived-macrophages (BMDMs) with wild-type (WT) and miR-223KO genotypes following stimulation with interleukin 4 (IL4) and with or without pioglitazone treatment (miR223KO-IL4+pio, miR223KO-IL4). The WT BMDMs were the controls (WT-IL4, WT-IL4+pio). B, A deficiency in miR-223 results in decreased gene expression of BMDMs upon stimulation with IL4 and pioglitazone. PGC1 α , peroxisome proliferator-activated receptor gamma, coactivator 1 alpha; Arg1, arginase 1. C, The expression of activation-related cell surface marker of WT BMDMs with miR-223 overexpression (miR223oe) following IL4 stimulation, with or without treatment with pioglitazone (miR223oe-IL4+pio, miR223oe-IL4). Data are presented as mean \pm SEM (n=3). * P <0.05, ** P <0.001. Means with different superscript letters are significantly different (P <0.05).

We confirmed the stimulatory effects of PPAR γ activation on the expression of miR-223 in M2 macrophages by demonstrating increased expression of miR-223 in BMDMs treated with pioglitazone and IL4 compared to control M2 cells (Figure 3.6A). This result was consistent with the observed increase in miR-223 expression in ATMs of mice treated with pioglitazone (Figure 3.3B), suggesting that PPAR γ may directly regulate miR-223 expression. Therefore, we screened 4 kilobases (kb) upstream of the miR-223 precursor gene (pre-miR-223) with JASPAR algorithms (<http://jaspar.binf.ku.dk/>) to identify PPAR γ response elements (PPREs).¹⁹³ There were eight predicted classical PPREs (AGG(A/T)CA) within the upstream region of pre-miR-223.¹⁹⁴⁻¹⁹⁶ The ChIP assay was used to further investigate PPAR γ interactions with the PPREs. The protein/DNA complexes were stabilized by formaldehyde crosslinking and isolated from BMDMs stimulated with IL4 and pioglitazone, and the sonicated DNA fragments (200-500bp) were immunoprecipitated using an antibody against PPAR γ . The enrichment of PPREs (-3816 to -3811, -3450 to -3445, or -2248 to -2243, relative to the 5' end of pre-miR-223 coding region) within 4kb upstream region of miR-223 precursor gene was verified by quantitative PCR with tiled primer pairs for determination of the precise location of each PPRE (Figure 3.6B).

Next, to determine the function of the PPRES as direct binding sites for PPAR γ , we generated a luciferase reporter construct harboring the PPRES (-3849 to -2117, relative to the 5' end of pre-miR-223 coding region) upstream of a SV40 promoter-luciferase expression cassette. After being transfected into macrophage cell line RAW264.7, the luciferase activity produced from the recombinant construct bearing PPRES was significantly increased compared to the control vector in the presence of IL4 (Figure 3.6C). Supplementation of pioglitazone further enhanced the luciferase activity generated from the PPRES-inserted constructs, but not the control vector in the transfected RAW264.7 cells stimulated by IL4 (Figure 3.6C). Thus, these results demonstrate that these three PPRES are the direct binding sites for PPAR γ and that miR-223 is a PPAR γ -regulated gene in M2 macrophages.

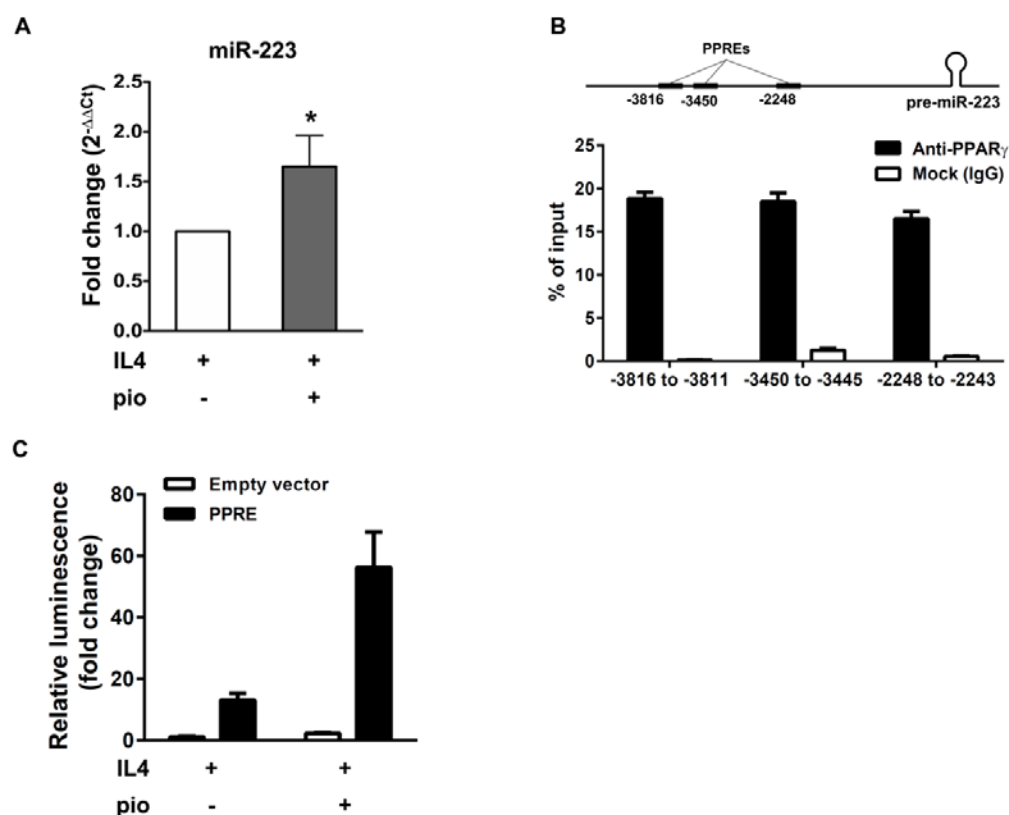


Figure 3.6 The expression of miR-223 is induced in PPAR γ -dependent activation of M2 macrophages. A, miR-223 expression in bone marrow derived macrophages (BMDM) increased in the presence of interleukin (IL) 4 (M2) with or without treatment with pioglitazone. B, After formaldehyde crosslinking and sonication, the protein/DNA complexes were immunoprecipitated with antibody against PPAR γ . The enrichment of potential PPAR γ response elements (PPREs) upstream of the miR-223 precursor gene (pre-miR-223) was validated by quantitative PCR using tiled primer pairs to determine the precise location of each PPRE. C, Luciferase activity of a reporter construct harboring the upstream region (-3849 to -2117 relative to the 5' end) of pre-miR-223 or empty vector in the presence of IL4 with or without treatment with pioglitazone. Data are presented as mean \pm SEM (n=3). * P <0.05, ** P <0.001.

***Rasa1* and *Nfat5* are genuine miR-223 target genes in PPAR γ -dependent activation of M2 macrophages**

MicroRNAs exert their biological functions by either blocking translation and/or inducing degradation of the target mRNAs by base-pairing to the recognition sites.^{135,136} We previously identified a pro-diabetic gene *Pknox1* as a bona fide miR-223 target gene that plays critical roles in modulating the function of miR-223 in M1 macrophages.⁹³ However, pioglitazone treatment did not affect expression of *Pknox1* in M2 cells (Figure 3.7A), suggesting that other miR-223 target genes are required for the PPAR γ -miR-223 regulatory axis for M2 responses. Thus, using prediction algorithms, TargetScan Mouse 6.2 and PicTar,^{173,174} we screened more than 20 predicted target candidates harboring miR-223 target sites. Among these, *Rasa1* and *Nfat5* were identified as genuine miR-223 target genes, as evidenced by suppression of their luciferase activities in the presence of miR-223 (Figure 3.7B). In contrast, mutation of the miR-223 binding site in the 3'-UTR of *Rasa1* and *Nfat5* prevented the inhibition of luciferase activity by miR-223 (Figure 3.7B). RT-PCR analysis revealed that expression of *Rasa1* and *Nfat5* were inversely correlated with miR-223 abundance in ATMs of lean mice (Figure 3.7C). Interestingly, the abundance of *Rasa1* and *Nfat5* was less in M2 WT BMDMs, and pioglitazone treatment further suppressed their expression in M2 WT macrophages (Figure 3.7D). These results suggest the potential roles of *Rasa1* and *Nfat5* in regulating PPAR γ -dependent M2 macrophage activation.

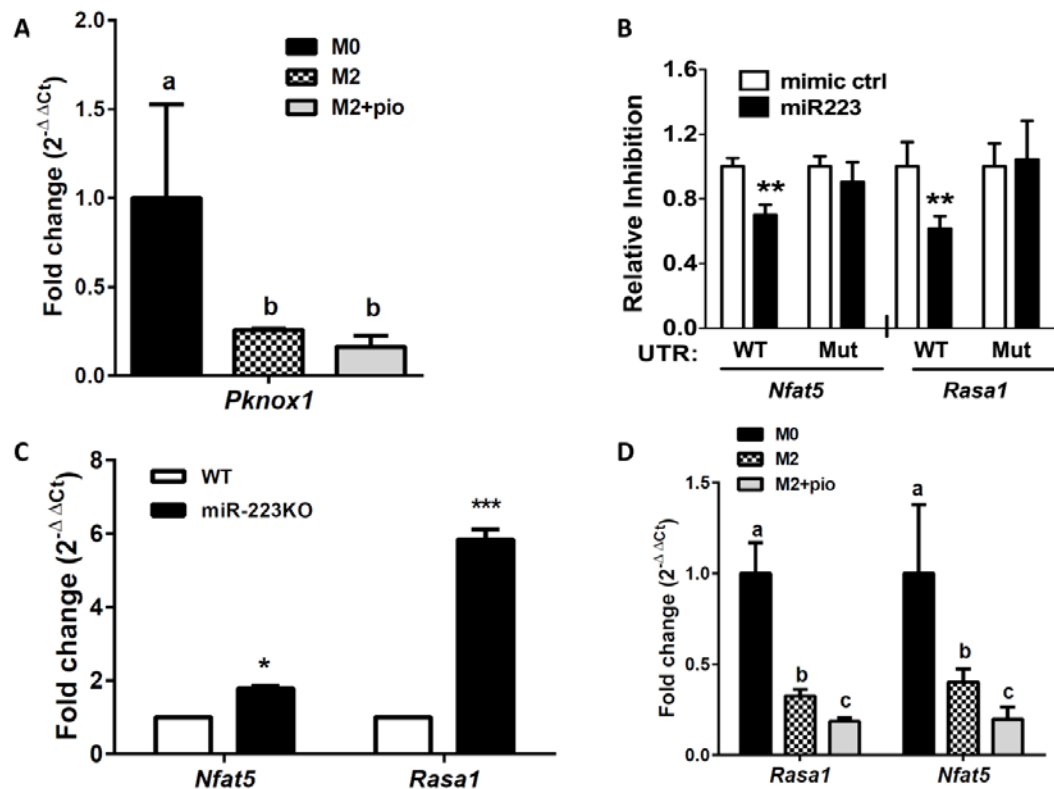


Figure 3.7 *Rasa1* and *Nfat5* are genuine miR-223 target genes. A, The expression of *Pknox1* in bone marrow derived macrophages (BMDM) was decreased in response to stimulation with interleukin 4 (IL4) (M2) compared to native BMDMs (M0), but pioglitazone (pio) treatment did not further reduce the expression of *Pknox1* in M2 macrophages (M2+pio). B, The assay with reporter constructs containing a 3' untranslated region (UTR) with wild type (WT) or mutated miR-223 binding site of target genes (mut) identified *Nfat5* and *Rasa1* as the miR-223 target genes. C, The RT-PCR analysis indicated that abundance of *Nfat5* and *Rasa1* in adipose tissue macrophages (ATMs) was greater in miR-223knockout (miR-223KO) lean mice compared to the WT lean mice. D, Expression of miR-223 target genes *Rasa1* and *Nfat5* M2 macrophages was decreased in M2 macrophages compared to M0 cells, and there were further inhibition on the expression of these genes in response to pioglitazone treatment. Data are presented as mean \pm SEM (n=3). * P <0.05, ** P <0.001, *** P <0.0001. Means with different superscript letters are significantly different (P <0.05). *Rasa1*, RAS p21 protein activator (GTPase activating protein) 1. *Nfat5*, nuclear factor of activated T-cells 5.

We next examined the impact of miR-223 target genes *Rasa1* and *Nfat5* on PPAR γ -mediated activation of M2 macrophages. Utilizing shRNA constructs, the expression of the miR-223 target genes, *Nfat5* and *Rasa1*, in miR-223KO BMDMs was significantly decreased compared to that for miR-223KO BMDMs transfected with an empty vector (Figure 3.8A). The expression of an M2 activation-related gene, arginase 1, increased after knockdown of *Rasa1* or *Nfat5* in miR-223KO BMDMs (Figure 3.8B). In addition, knockdown of these miR-223 target genes resulted in enhanced M2 activation in response to IL4 stimulation, as evidenced by significant increases in expression of activation-related cell surface markers CD69 and CD86 (Figure 3.8C). Taken together, these results demonstrate that miR-223 target genes, *Rasa1* and *Nfat5*, play critical roles in the regulatory axis of PPAR γ -miR-223 to control activation of M2 macrophages.

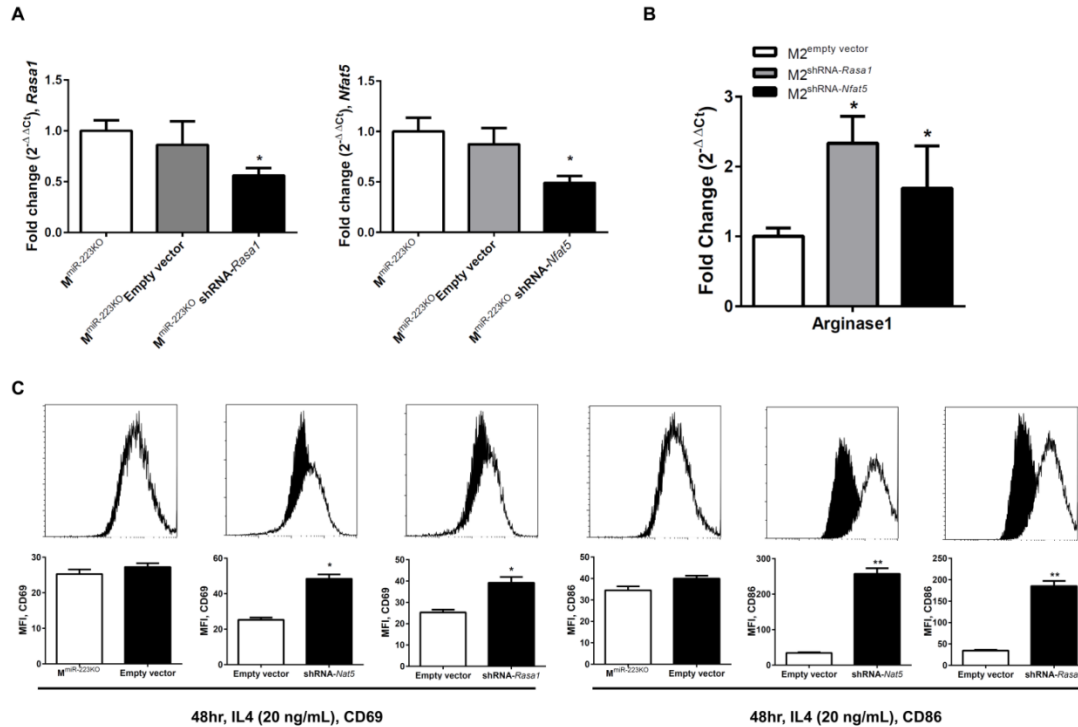


Figure 3.8 miR-223 controls M2 macrophage activation by modulating expression of multiple target genes. A, Knockdown of miR-223 target genes *Rasa1* and *Nfat5* expression in miR-223KO bone marrow derived macrophages (BMDM) by short hairpin RNA (shRNA) assay. B, The expression of arginase 1 which is associated with activation of M2 macrophages increased in response to knockdown of miR-223 target genes *Rasa1* or *Nfat5*. C, Expression of macrophage activation-related cell surface markers CD69 and CD86 in BMDMs increased following knockdown of miR-223 target genes *Rasa1* or *Nfat5* in the presence of IL4. Data are presented as mean \pm SEM (n=3). * P <0.05, ** P <0.001. *Rasa1*, RAS p21 protein activator (GTPase activating protein) 1. *Nfat5*, nuclear factor of activated T-cells 5.

Discussion

Adipose tissue macrophages exert profound impacts on the homeostasis of adipose tissues by responding to distinct stimuli with heterogeneous activation phenotypes.^{35,197,198} The ATMs with an M1 phenotype are crucial for promoting chronic inflammation induced by obesity and clearance of apoptotic adipocytes;^{42,199,200} whereas M2 ATMs are major coordinators of adipose tissue remodeling and repair with anti-inflammatory features.^{201,202} In the context of obesity, there is a dramatic increase in the infiltration of macrophages into white adipose tissues which account for more than 40% of the stromal cell population in visceral fat depots in obese individuals.^{43,60} Under obesity-induced stress, ATMs not only increase in number, but also display an increased M1/M2 ratio for macrophages which result in an overall enhancement of inflammation in adipose tissues leading to the development of systemic insulin resistance.^{80,176} Polarization of ATMs is tightly regulated by a well-orchestrated network in which PPAR γ plays a central role in controlling activation of ATMs.²⁰³ In addition, epigenetic regulators, including microRNAs, exert an additional layer of regulation in the regulatory network.⁵⁷ Our previous study demonstrated that miR-223 is a potent regulator that controls the polarization of ATMs in response to distinct signals in the microenvironment.⁹³ However, the interplay between microRNAs and PPAR γ -regulated signaling pathway is unclear. Results of this study provided clear evidence for a key role for the PPAR γ -miR-223 axis in modulating activation of M1 and M2 responses in both in vivo and ex vivo

systems. Our findings revealed that PPAR γ acts as an enhancer to promote the expression of miR-223 in ATMs upon M2 activation, and that miR-223 is a critical mediator for PPAR γ -dependent effects for activation of ATMs.

Upon stimulation with Th2 cytokines such as IL4 and IL13, activation of the PPAR γ -mediated signaling pathway is essential for triggering M2 responses such as the induction of expression of arginase 1 and IL10 in ATMs.⁶⁶ PPAR γ can also act as a transcriptional repressor of the inflammatory responses of macrophages through interrupting activity of the NF κ B signaling pathway.⁶⁸⁻⁷⁰ Odegaard et al. reported that knockout of macrophage-specific PPAR γ impairs M2 responses of ATMs, subsequently exacerbating obesity-associated insulin resistance.⁶⁶ Results of our study demonstrated that ligand-induced activation of the PPAR γ -mediated signaling pathway suppressed activation of proinflammatory M1 ATMs (Figure 3.3A). Thus, a decrease in the inflammatory responses of ATMs attenuates adipose tissue inflammation in the context of obesity.^{80,159,178} In this study, we detected suppressive effects of PPAR γ on inflammatory responses of ATMs in adipose tissue and a decrease in systemic insulin resistance (Figure 3.1).

The myeloid cell-specific miR-223 exhibits significant epigenetic functions to regulate macrophage activation through post-transcriptional suppression of expression of its target genes.^{93,204-206} The expression of miR-223 is increased significantly in response to M2 activation, but did not change during activation of

M1 macrophages.⁹³ More importantly, the induction of miR-223 favors activation of anti-inflammatory M2 macrophages.⁹³ Conversely, miR-223 deficient macrophages exhibit hypersensitivity in M1 responses induced by LPS stimulation and delayed responses to Th2 cytokine stimulation. In addition, in our previous study, *Pknox1* was validated as a miR-223 target gene mediating effects of miR223 to activate macrophages.⁹³ In the diet-induced obesity mouse model, we observed that loss of miR-223 resulted in an increase in the proportion of M1 ATMs in HFD-fed mice compared to HFD-WT mice which confirms results of our previous study.⁹³ Given that expression of miR-223 is critical for M2 macrophage responses, we identified PPAR γ as a key regulator of expression of miR223. Using ChIP-qPCR, we confirmed that PPAR γ interacts with three PPRES within the upstream region of the miR-223 precursor gene in macrophages (Figure 3.6B). Further, results of the luciferase reporter assay verified direct interactions between PPAR γ and the three PPRES (Figure 3.6C), and demonstrated critical roles for the PPRES for induction of PPAR γ -induced expression of miR-223 in macrophages. This accounts for the increased expression of miR-223 in ATMs in response to activation of PPAR γ under obesity-induced stress. In addition, PPAR γ exerts profound effects on various cell types, such as adipocytes, to modulate expression patterns of microRNAs during adipogenesis.^{207,208}

We further demonstrated that miR-223 is a critical downstream component in the PPAR γ -dependent signaling pathway controlling ATM

activation. Mice with miR-223 deficiency display impaired beneficial outcomes after administration of a PPAR γ agonist, pioglitazone, compared to wild type control mice, suggesting miR-223 is a crucial mediator of PPAR γ action. The important role of miR-223 in the PPAR γ -regulated signaling network was further confirmed in the present study using the *ex vivo* macrophage polarization system. Deletion of miR-223 in macrophages delayed M2 activation which was not rescued by administration of the PPAR γ agonist pioglitazone. In addition, overexpression of miR-223 further enhanced PPAR γ -dependent M2 macrophages with respect to cell-activation related surface markers, cytokine production, and gene expression. We also found that *Rasa1* and *Nfat5* were novel miR-223 target genes that have critical roles in miR-223 function on M2 macrophage activation, as evidenced by enhanced M2 responses after knockdown of expression of these target genes. Therefore, these results suggest that the regulatory role of miR-223 in the PPAR γ -mediated signaling pathway depends on a network involving the novel target genes *Rasa1* and *Nfat5*.

In this study, we have showed that the PPAR γ -miR-223 regulatory axis is crucial for controlling activation status of ATMs and subsequent adipose tissue inflammation and insulin sensitivity (Figure 3.9). miR-223 and its target genes *Rasa1* and *Nfat5* act as the critical downstream components of the PPAR γ -mediated cell signaling pathway that regulates ATM activation. Furthermore, identification of novel miR-223 target genes *Nfat5* and *Rasa1* in this context

provides new gene targets for development of drugs with the potential to mitigate obesity-related diseases.

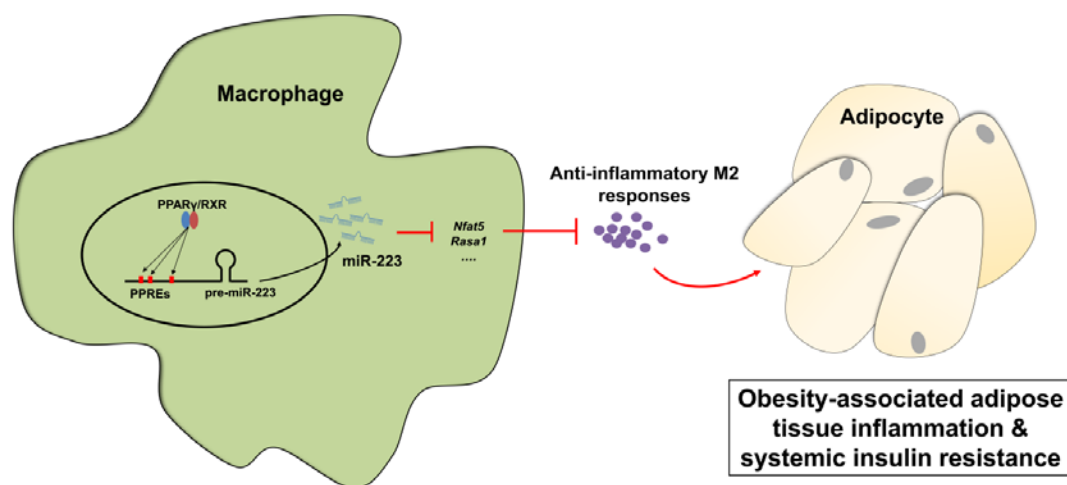


Figure 3.9 Schematic model of the PPAR γ -miR-223 regulatory axis in controlling activation of M2 macrophages and subsequent adipose tissue inflammation and systemic insulin resistance under the stress of obesity. PPAR γ acts an enhancer to promote the expression of miR-223 through interacting with PPAREs. miR-223 is required for PPAR γ action in inducing M2 responses through controlling the expression of target genes *Nfat5* and *Rasa1*. The PPAR γ -miR-223 axis-mediated M2 responses can attenuate obesity-associated adipose tissue inflammation and insulin resistance. PPAR γ , peroxisome proliferator-activated receptor gamma. PPARE, PPAR γ response element. RXR, retinoid X receptor. *Rasa1*, RAS p21 protein activator (GTPase activating protein) 1. *Nfat5*, nuclear factor of activated T-cells 5.

CHAPTER IV

INTERFERON TAU ALLEVIATES OBESITY-INDUCED ADIPOSE TISSUE INFLAMMATION AND INSULIN RESISTANCE BY REGULATING MACROPHAGE POLARIZATION*

Chronic adipose tissue inflammation is a hallmark of obesity-induced insulin resistance and anti-inflammatory agents can benefit patients with obesity-associated syndromes. Currently available type I interferons for therapeutic immunomodulation are accompanied by high cytotoxicity. Therefore, in this study, we have examined anti-inflammatory effects of interferon tau (IFNT), a member of the type I interferon family with low cellular toxicity even at high doses. Using a diet-induced obesity mouse model, I observed enhanced insulin sensitivity in obese mice administered IFNT compared to control mice, which was accompanied by a significant decrease in secretion of proinflammatory cytokines and an increase in anti-inflammatory macrophages (M2) in adipose tissue. Further investigations revealed that IFNT is a potent regulator of macrophage activation that favors anti-inflammatory responses as evidenced by activation of associated surface antigens, production of anti-inflammatory cytokines, and activation of selective cell signaling pathways. Thus, my study demonstrates, for the first time, that IFNT can significantly mitigate obesity-associated systemic insulin resistance and tissue inflammation by controlling

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macrophage polarization, and thus IFNT can be a novel bio-therapeutic agent for treating obesity-associated syndromes and type 2 diabetes.

Introduction

Obesity and its associated metabolic abnormalities, including insulin resistance and cardiovascular disorders, have reached epidemic proportions. Chronic low degree adipose tissue inflammation, accompanied by enhanced immune cell infiltration, is a hallmark of obesity and a crucial contributor to the pathogenesis of insulin resistance and metabolic diseases.^{5,44,80,107,110,139,142} The infiltrated immune cells play critical roles in modulating obesity-associated adipose tissue inflammation. Among them, macrophages account for up to 50% of the stromal cell population in adipose tissues of obese individuals and are critical regulators of adipose tissue functions.^{44,60,160,209,210} In addition, adipose tissue macrophages (ATMs) undergo a phenotypic switch from anti-inflammatory status (M2) in the adipose tissues of lean individuals to a proinflammatory (M1) status in adipose tissues of obese subjects, which results in the development of tissue inflammation and systemic insulin resistance.¹⁷⁶ The classic proinflammatory responses of ATMs (M1) depend on Toll-Like Receptors (TLRs) and activation of nuclear factor κ B (NF κ B)/c-Jun N-terminal kinase (JNK), leading to the production of inflammatory cytokines.²¹¹ In contrast, activation of M2 ATMs leads to recruitment of peroxisome proliferator-activated receptor γ (PPAR γ) or other transcription factors resulting in an anti-inflammatory

status.^{66,161,212,213} Our recent study revealed that other molecules, such as microRNAs, can exhibit profound regulatory functions on macrophage polarization.⁹³ Activation of M2 macrophages can improve systemic insulin sensitivity and protect against development of cardiovascular diseases and type 2 diabetes.^{214,215}

Clinical studies show the anti-inflammatory treatments can benefit patients with systemic insulin resistance.^{3,216,217} Type I interferons have been used as anti-inflammatory therapies by suppressing production of inflammatory cytokines such as interleukin (IL)-1 β and tumor necrosis factor α (TNF- α).²¹⁸⁻²²⁰ However, severe cell toxicities associated with currently available type I IFNs, such as IFN- α and - β (IFNA and IFNB),^{221,222} dramatically hinder their clinical application. Recent studies revealed that a member of type I IFN family, interferon tau (IFNT), exerts potent immunomodulatory effects with very low cytotoxicity even at high dosages, which provides support for a clinical application of IFNT.

IFNT is a type I interferon produced exclusively by mononuclear trophoblast cells of conceptuses of ruminant species and plays a central role for successful implantation and establishment of pregnancy.¹²⁸ IFNT shares high protein structural similarities with other members of the type I interferon family, such as IFNA and IFNB and particularly interferon omega (IFNW).²²³ Compared with IFNA and IFNB that have been applied as clinical therapeutics, IFNT lacks

their cytotoxicity even at high concentrations.^{224,225} IFNT can shift the immune profile from an inflammatory to an anti-inflammatory phenotype to mitigate against autoimmune-associated diseases.^{131,134} Here, we show that IFNT is a potent regulator of macrophage polarization that significantly suppresses obesity-associated adipose tissue inflammation and ameliorates systemic insulin resistance. Thus, this study provides important evidence, for the first time, toward understanding the regulatory mechanism of IFNT action in the context of obesity and indicates potential clinical applications for IFNT in treating obesity-associated metabolic syndrome

Materials and Methods

Animals

Male C57BL/6J mice, 6 weeks of age, were used for diet feeding and bone marrow isolation and macrophage activation analyses. All mice were maintained on a 12-/12-hour light-dark cycle and fed *ad libitum*. To induce obesity, mice were fed a high-fat diet (HFD; Cat. No. D12492, Research Diets, Inc) for 12 weeks. Mice fed on a low-fat diet (LFD; Cat. No. D12450B, Research Diets, Inc) served as controls. In the IFNT treatment, mice received recombinant IFNT via drinking water (8 µg/kg body weight/day) for 12 weeks.³ The IFNT (30-45 ng/mL) delivered orally in drinking water (1.5 mL/10 g body weight/day) was detected in plasma of mice using a highly specific and sensitive radioimmunoassay (Figure 4.1).²²⁶ Glucose metabolism and insulin sensitivity of

LFD and HFD mice were evaluated by measuring concentrations of glucose and insulin in plasma, and conducting glucose tolerance and insulin tolerance tests. The glucose tolerance and insulin tolerance tests were performed during week 12 of the study, and there was a 6-day interval between these two tests. In addition, the glucose tolerance and insulin tolerance tests were performed between 8:00 AM and 10:00 AM for all mice. Blood samples were collected from the tail vein of un-anesthetized mice and stored in EDTA-coated tubes, and plasma was harvested after the blood was centrifuged at 1,000 x g for 15 min at 4°C. At the end of the study, all mice were euthanized between 8:00 AM and 10:00 AM by exposure to a high concentration of carbon dioxide (CO₂). In this study, we combined the visceral adipose tissues (VAT; retroperitoneal fat, perirenal fat, mesenteric fat, perigonadal fat) for isolation of mature adipocytes, measurement of gene expression, immunohistochemistry, and evaluation of immune cell infiltration. The VAT from mice were fixed in fresh 4% paraformaldehyde and used for immunohistochemical analysis or snap frozen in liquid nitrogen and stored at -80°C. All study protocols were approved by the Institutional Animal Care and Use Committee of Texas A&M University.

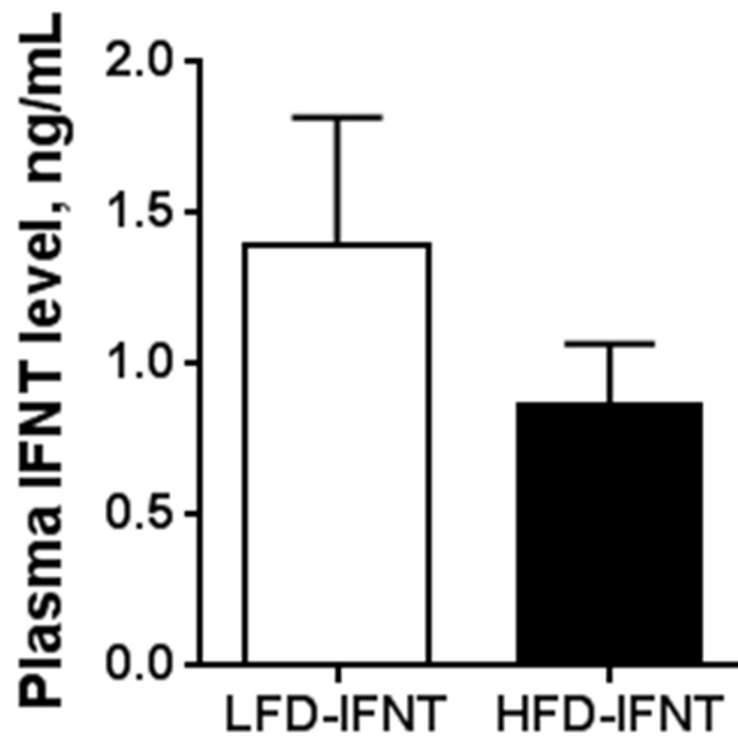


Figure 4.1 Plasma level of interferon tau (IFNT). Concentrations of IFNT in plasma of mice fed a high fat diet were determined by radioimmunoassay after 12 weeks of treatment with IFNT in drinking water (8 $\mu\text{g/kg/day}$). Data are presented as mean \pm SEM (n=4-5).

Mature adipocyte isolation and ex vivo analysis of insulin signaling

Visceral adipose tissue was minced and digested in Hank's Balanced Salt Solution (HBSS; Cat. No. 21-022-CM, Corning®) digestion buffer containing 1 mg/mL collagenase II, 1% BSA and 100 mM HEPES for 40 min at 37°C. After passing through a 250 µm nylon mesh, the stromal cells and mature adipocytes were separated by centrifuging at 1,500 x g for 5 min at 4°C. For ex vivo analysis of insulin signaling, the mature adipocytes were collected and stimulated with or without 100 nM insulin in Dulbecco's Modified Eagle Medium/Nutrient F-12 Ham (DMEM/F12; Cat. No. 16777-133, VWR International) for 15 min, followed by cell lysis in Radio-Immunoprecipitation Assay (RIPA; Cat. No. 9806, Cell Signaling) buffer containing a protease/phosphatase inhibitor cocktail (Cat. No. 5872S, Cell Signaling).

Bone marrow isolation and macrophage differentiation

Bone marrow-derived macrophages (BMDMs) were obtained as described previously.¹⁵⁹ After red blood cell lysis (Table A-4), bone marrow cells were seeded at 2×10^6 cells/mL with Iscove's Modified Dulbecco's Medium (IMDM; Cat. No. 16777-182, VWR International) medium containing 10% FBS and 15% L929 culture supernatant as a source of granulocyte macrophage colony-stimulating factor (GM-CSF) for differentiation of bone marrow cells to monocytes. After 7 days, the formation of mature monocytes was evaluated by flow cytometry using antibodies against CD11b and F4/80 cell surface antigens.

Macrophage polarization analysis

Bone marrow derived macrophages were stimulated by lipopolysaccharide (LPS; 100 ng/mL) for M1 activation or IL-4 (20 ng/mL) for M2 activation. To test the dosage effects of IFNT, BMDMs were treated with IFNT at 10,000, 5,000, or 1,000 anti-viral units (AVU)/mL for 48 h in the presence of LPS or IL-4 (Figure 4.2). After 48 h of stimulation, BMDMs were examined for activation of expression of associated surface antigens CD69, CD80, and CD86 using flow cytometry.

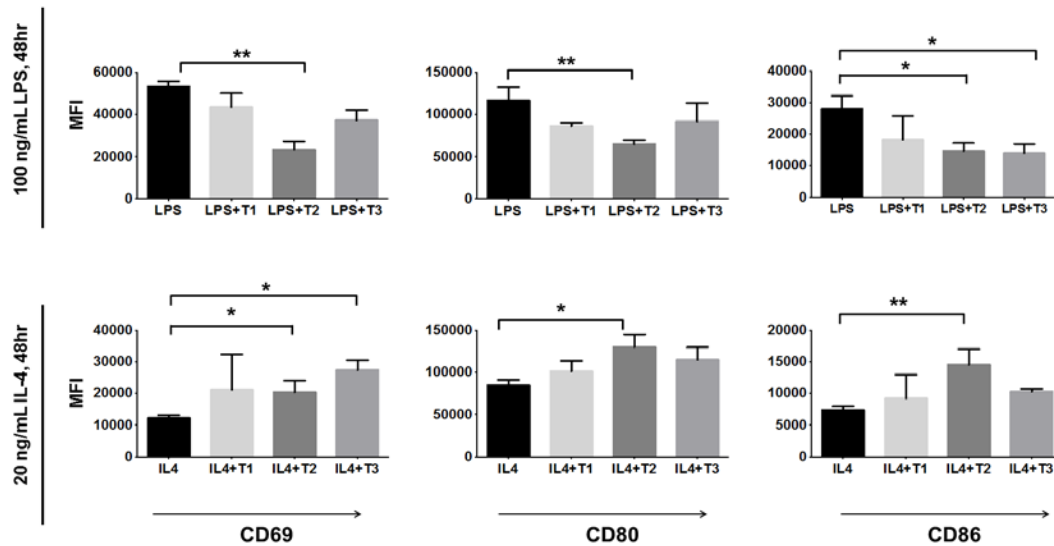


Figure 4.2 Dosage effect of interferon tau (IFNT) on macrophage polarization. Bone marrow-derived macrophages (BMDMs) were stimulated with lipopolysaccharide (LPS, 100 ng/mL) or interleukin 4 (IL4, 20 ng/mL). In addition, BMDMs were treated with IFNT at 1,000 (T1), 5,000 (T2), 10,000 (T3), or 0 anti-viral units (AVU)/mL. After 48 hours, the surface makers CD69, CD80, and CD86 of BMDMs by were analyzed using flow cytometry to reveal significant effects of treatment. Data are presented as mean \pm SEM (n=3). * P <0.05, ** P <0.001. MFI, medium fluorescence intensity.

Flow cytometry analysis

The Vascular stromal cells (VSC) of VAT and BMDMs were stained with fluorescence-conjugated antibodies to detect cell lineages in VAT or their activation. B cells were detected with antibodies against cell surface antigens B220 (Cat. No. 48-0452-82, eBioscience), CD19 (Cat. No. 17-0193-82, eBioscience), CD5 (Cat. No. 11-0051-82, eBioscience) and CD43 (Cat. No. 12-0431-82, eBioscience); T cells were detected with antibodies against cell surface antigens CD4 (Cat. No. 11-0041-82, eBioscience) and CD8 (Cat. No. 17-0081-82, eBioscience); macrophage subtypes and activation of macrophages were detected using antibodies against cell surface markers F4/80 (Cat. No. 11-4801-82, eBioscience), CD11b (Cat. No. 45-0112-82, eBioscience), CD206 (Cat. No. 141706, Biolegend), CD11c (Cat. No. 12-0114-82, eBioscience), CD80 (Cat. No. 17-0801-82, eBioscience), CD69 (Cat. No. 45-0691-82, eBioscience), and CD86 (Cat. No. 11-0862-82, eBioscience). Phosphorylated signal transducers and activators of transcription 1 (STAT1; Cat. No. 612564, BD Biosciences) and STAT3 (Cat. No. 557814, BD Biosciences) of BMDMs were detected using an intracellular staining assay. Flow cytometry analysis was performed using Accuri C6 (BD Bioscience), and results were analyzed using Flowjo or Accuri C6 software (BD Bioscience).

Immunohistochemistry

Tissues collected from HFD-fed mice were fixed and stained with antibodies against cell surface markers F4/80 (Cat. No. ab6640, abcam), B220 (Cat. No. ab10558, abcam), and CD3 (Cat. No. ab16669, abcam) to detect macrophages, B cells, and T cells, respectively. Immunoglobulin (IgG) protein was used as the negative control. Images were captured using a Zeiss Stallion Dual Detector Imaging System with Intelligent Imaging Innovations Software (Carl Zeiss).

Western blotting

After homogenization of VATs using a BeadBugTM microtube homogenizer (Benchmark Scientific), total protein was extracted from VAT homogenate using RIPA buffer, and protein concentrations were determined using the Bradford assay. Proteins were separated on PROTEAN[®] TGX Stain-FreeTM Precast Gel (Cat. No. 456-8044, Bio-Rad) and transferred to a polyvinylidene fluoride (PVDF) membrane followed by detection of the respective antigens using the appropriate antibodies. Activation of the NFκB pathway in adipose tissues was evaluated using antibodies against p65 (Cat. No. 4764, Cell Signaling) and phosphorylated p65 (Pp65; Cat. No. 3033, Cell Signaling).

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted from adipose tissues or BMDMs using the Trizol extraction protocol according to the manufacturer's instructions. Gene expression analysis was performed using the iScript One-Step RT-PCR kit with SYBR Green (Cat. No. 170-8893, Bio-Rad) on Bio-Rad CFX384 (Bio-Rad). The data presented correspond to the mean of $2^{-\Delta\Delta C_t}$ from at least three independent experiments after being normalized to β -actin.

Bio-Plex protein expression assay

The concentrations of IL-1 β , TNF- α , IL-6, IL-10, and chemokine (C-C motif) ligand 2 (CCL2) in plasma were determined using Bio-PlexTM Cytokine Assay (Cat. No. M60-009RDPD, Bio-Rad). Concentrations of insulin in plasma were determined using the Bio-Plex Pro Mouse Diabetes Insulin set (Cat. No. 171-G7006M, Bio-Rad). The levels of total (Cat. No. 171-V60007M, Bio-Rad) and phosphorylated (Cat. No. 171-V50011M, Bio-Rad) JNK, and total (Cat. No. 171-V60001M, Bio-Rad) and phosphorylated (Cat. No. 171-V50001M, Bio-Rad) Akt in mature adipocytes were determined using the Bio-Plex Cell Signaling Magnetic Assays (Bio-Rad). These Bio-Plex assays were performed using the Bio-Plex MAGPIXTM multiplex reader (Bio-Rad). Results were analyzed using Bio-Plex Data ProTM software (Bio-Rad).

Data and statistical analyses

Results are expressed as means \pm SEM. Each data point derived from qRT-PCR assays represents an average of two technical replicates, and data were averaged over independently replicated experiments (n=3-4 independently collected samples) and analyzed using the Student's *t* test. The overall group-effect was analyzed for significance using two-way ANOVA and the Bonferroni post-test for each factor at each individual time. Data analyses were performed using Graphpad Prism version 6.0 software. A value of $P < 0.05$ was considered statistically significant.

Results

Interferon tau alleviates obesity-induced insulin resistance

To evaluate the effects of IFNT on obesity-associated inflammation and insulin resistance, we adopted a diet-induced obese mouse model. An effective IFNT dose was chosen based on previous studies.²²⁷ After 12 weeks feeding, IFNT treatment did not significantly affect body weight gain or food intake in either the HFD or LFD groups (Figure 4.3A, B). However, compared to the control HFD mice (HFD-Control mice), IFNT treatment (HFD-IFNT mice) decreased hyperglycemia and concentrations of insulin in blood (Figure 4.3C). In addition, HFD-IFNT mice had lower concentrations of glucose and insulin in blood after 16-h of fasting compared to the HFD-Control mice (Figure 4.3C). To evaluate the effects of IFNT on insulin sensitivity, mice were subjected to

glucose and insulin tolerance tests. Mice were fasted for 16 h and then injected with a single dose of glucose (2 mg of glucose per gram body weight) or insulin (1 U of insulin per kg body weight) followed by determination of concentrations of blood glucose at various time points (Figure 4.3D, E). With LFD mice, IFNT treatment did not alter concentrations of glucose or insulin in plasma; whereas HFD-IFNT mice had lower concentrations of glucose in plasma than HFD-Control mice (Figure 4.3D, E). Collectively, these results suggest that IFNT improves obesity-associated glucose metabolism and insulin sensitivity.

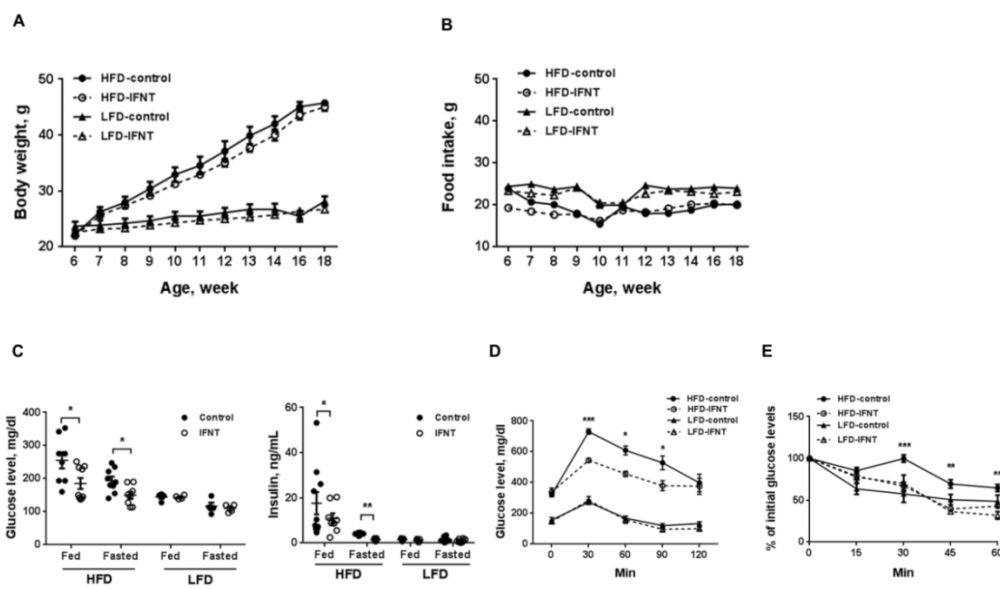


Figure 4.3 Interferon tau (IFNT) alleviates insulin resistance in mice fed a high-fat diet (HFD). A and B, Body weight and food intake of mice were monitored during a 12-week feeding period (n=9-10). C, Concentrations of glucose and insulin in plasma of control or IFNT-treated mice fed a HFD or fasted for 16 h were less than that of the HFD control mice but not in the mice fed a low-fat diet (LFD). Results of glucose tolerance test (D) and insulin tolerance test (E) indicated that IFNT treatment improved the insulin sensitivity of HFD mice (n=6). Data are presented as mean \pm SEM. * P <0.05, ** P <0.001, *** P <0.0001.

Interferon tau alleviates obesity-associated inflammation

There is compelling evidence for causal effects of obesity-associated chronic inflammation, especially in adipose tissues of obese individuals, and the pathogenesis of systemic insulin resistance.⁸⁰ Although HFD-IFNT and HFD-Control mice had similar degrees of adiposity (Figure 4.4A), IFNT treatment suppressed activation of NF κ B as evidenced by lower p65 phosphorylation and JNK pathway activity particularly decreased abundance of phosphorylated-JNK in VAT of HFD mice (Figure 4.4B, C). In addition, HFD-IFNT mice displayed lower levels of proinflammatory cytokines, namely IL-1 β , IL-6 and TNF- α , and increased expression of the anti-inflammatory cytokine IL-10 in adipose tissue compared to HFD-Control mice (Figure 4.5A). However, the expression of CCL-2 in adipose tissues isolated from HFD mice was not affected by IFNT treatment (Figure 4.5A). Expression of adiponectin, an adipokine negatively associated with obesity, was induced by IFNT treatment in HFD mice compared to the control group (Figure 4.5A).

We also detected decreased concentrations of CCL2 and TNF- α and elevated concentrations of IL-10 in plasma of HFD-IFNT mice compared to HFD-Control mice (Figure 4.5B). We further determined the impact of IFNT treatment on the insulin signaling pathway in adipose tissues of HFD mice. In the *ex vivo* analysis of insulin signaling, we detected an increase in the abundance of phosphorylated Akt protein in isolated mature adipocytes treated with insulin compared to that in mature adipocytes from mice that were not treated with insulin. This result indicates that insulin successfully activated the intracellular insulin signaling pathway in the isolated mature adipocytes. Interestingly, total Akt protein and its insulin-responsive phosphorylation were both greater in mature adipocytes isolated from HFD-IFNT mice than HFD-Control mice (Figure 4.5C). Taken together, our results suggest that IFNT treatment effectively modulates obesity-associated insulin resistance at least by suppressing tissue inflammation.

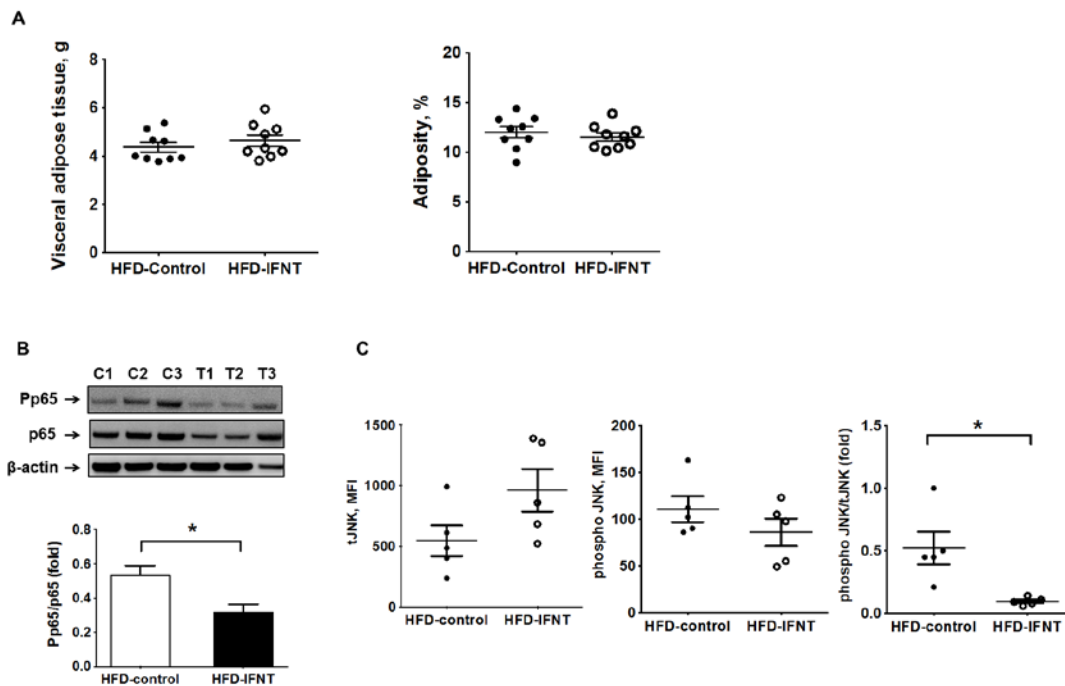


Figure 4.4 Interferon tau (IFNT) reduces obesity-associated adipose tissue inflammation. A, Visceral adipose tissues (VAT) weight and adiposity of mice after 12-week HFD feeding were not affected by treatment with IFNT. B, Nuclear factor-κB (NFκB) activation in VAT of mice fed a HFD was reduced by IFNT treatment based on results from western blotting with antibodies against p65 and phosphorylated p65 (Pp65; n = 3). C, control; T, IFNT. C, Activation of c-Jun N-terminal kinase (JNK) signaling pathway in VAT of mice fed a HFD was assessed by measuring fluorescent-labeled beads conjugated with antibodies against total JNK (tJNK) and phosphorylated JNK (phospho JNK) using the Bio-Plex® MAGPIX™ multiplex reader. Results indicated that IFNT treatment significantly reduced the activation of JNK signaling in VAT of obese mice. MFI, medium fluorescence intensity. Data are presented as mean ± SEM. * $P < 0.05$.

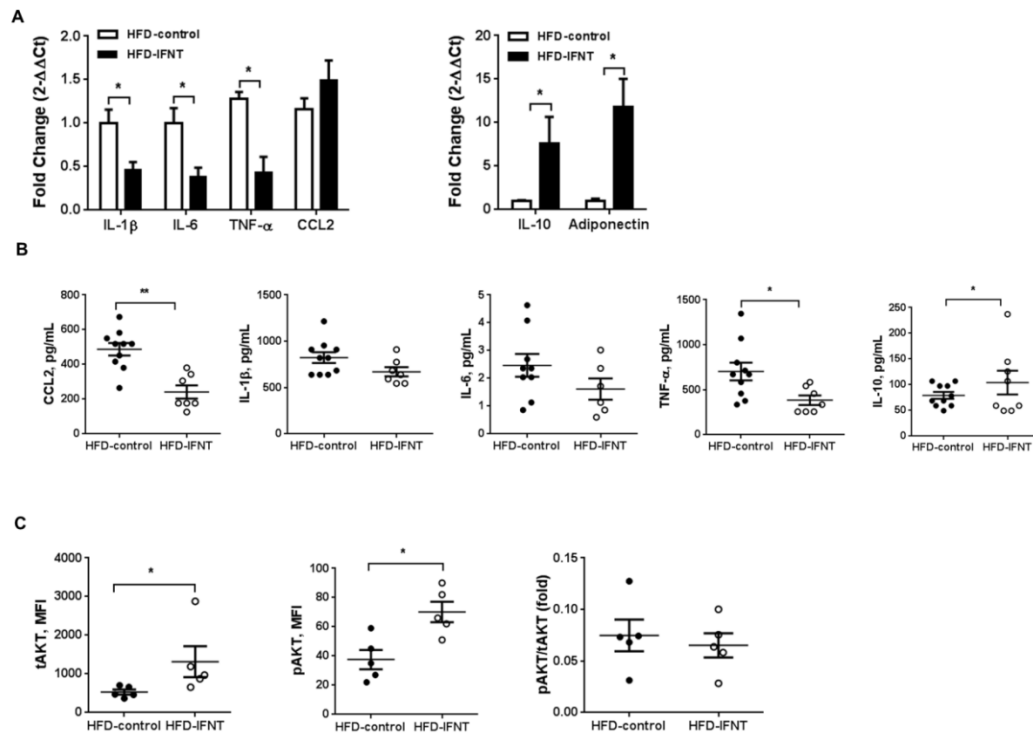


Figure 4.5 Oral administration of interferon tau (IFNT) alters the cytokine profile and insulin signaling. A, Expression of genes encoding interleukin 1 beta (IL-1 β), IL6, and tumor necrosis factor alpha (TNF α) was inhibited, while expression of IL10 and adiponectin increased in response to treatment with IFNT. B, Concentrations of IL-1 β , IL-6, TNF- α , CCL2, and IL-10 in plasma of mice fed a high fat diet (HFD) were measured using the Bio-PlexTM Cytokine Assay (Bio-Rad). The results indicated that IFNT treatment decreased concentrations of CCL2 and TNF α but increased IL10, compared to the control. C, Adipose tissue insulin signaling. After 16-h fasting, mature adipocytes were collected from VAT and treated with insulin (100 nM) for 15 min. Total AKT (tAKT) and phosphorylated AKT (pAKT) protein in adipocytes increased in response to IFNT, but in a proportional manner so that the ratio was not different as determined using Bio-Plex Cell Signaling Magnetic Assays (Bio-Rad). Data are presented as mean \pm SEM. * $P < 0.05$.

Interferon tau regulates macrophage activation in adipose tissues of HFD mice

To further understand the impact of IFNT on adipose tissue immune cell populations that are major contributors to adipose tissue inflammatory status, we examined the relative proportions of T cells, B cells and macrophages in stromal cells of VAT from HFD-IFNT and HFD-Control mice. We first performed immunohistochemical staining on VAT isolated from both HFD-IFNT and HFD-Control groups with antibodies against F4/80 (macrophages), B220 (B cells) and CD3 (T cells). The results indicated that total numbers of macrophages, B cells and T cells infiltrated into VAT are comparable in HFD-IFNT and HFD-Control mice (Figure 4.6A). This was confirmed by flow cytometry assays with the same set of antibodies. Consistent with results of immunohistochemical staining, the proportions of total macrophages (F4/80⁺CD11b⁺), B cells (B220⁺) and T cells (CD4⁺ or CD8⁺) were not significantly different between IFNT treated and control mice on either the HFD (Figure 4.6B) and the LFD (Figure 4.7). Surprisingly, the distribution of macrophage subpopulations, i.e. M1 and M2 macrophages, was significantly altered by IFNT treatment. Compared to the HFD-Control mice, HFD-IFNT mice displayed dramatically decreased proinflammatory M1 macrophages (F4/80⁺CD11b⁺CD206⁻CD11c⁺; Figure 4.6C) and a significant increase in anti-inflammatory M2 macrophages (F4/80⁺CD11b⁺CD206⁺CD11c⁻; Figure 4.6C) which suggests a regulatory role for IFNT in macrophage polarization.

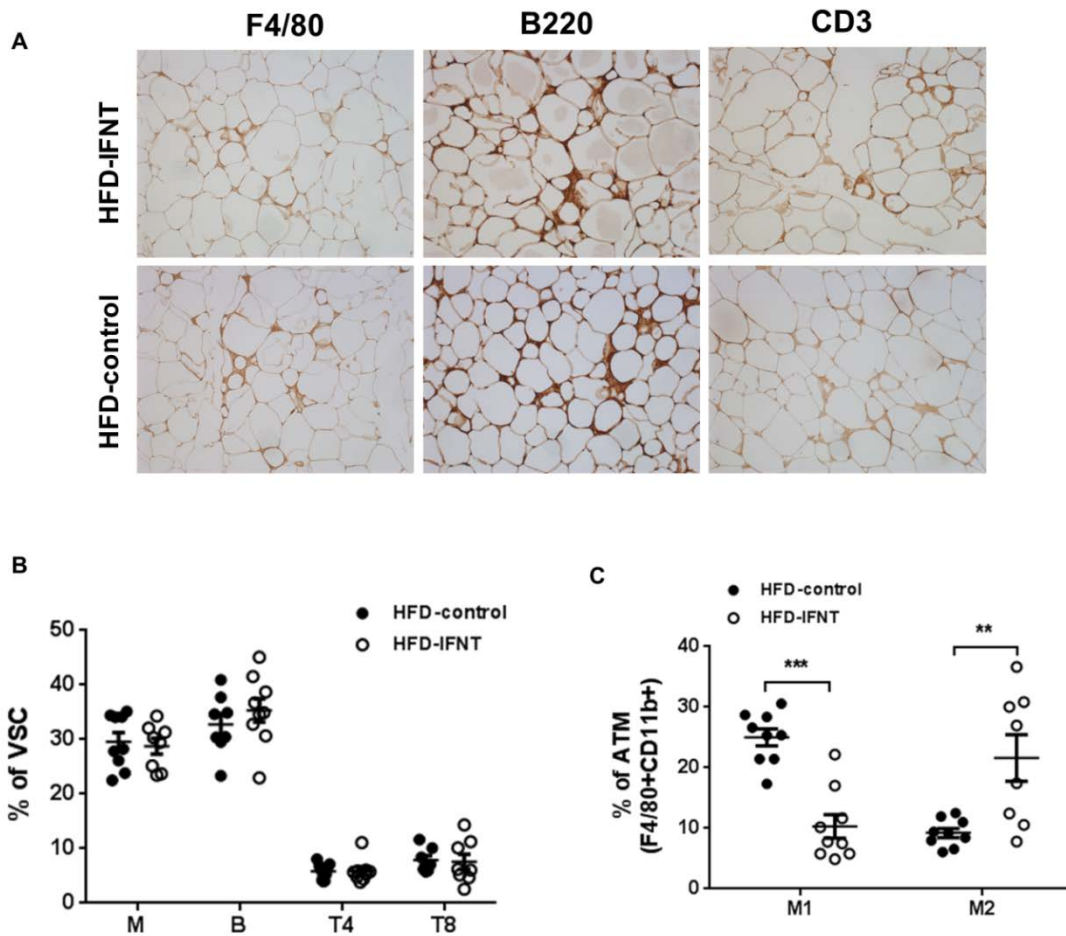


Figure 4.6 Interferon tau (IFNT) regulates macrophage activation in adipose tissues of mice fed a high fat diet (HFD). A, Sections of adipose tissue from mice fed a HFD mice were stained with antibodies against F4/80, B220 and CD3 for macrophages, B cells and T cells, respectively. The results suggested that IFNT treatment had minimal effects on the infiltration of these immune cells into visceral adipose tissue (VAT). B, Macrophage (M), B cell (B), CD4⁺ T cell (T4) and CD8⁺ T cell (T8) infiltration into VAT of mice fed a HFD was not affected by IFNT based on flow cytometry analyses using antibodies against F4/80, CD11b, B220, CD4 and CD8. C, Macrophage subtypes in visceral fat stromal cells (VSC) of VAT were analyzed by flow cytometry using antibodies against F4/80, CD11b, CD11c and CD206. The results indicated a decrease in M1 macrophages and an increase in M2 macrophages after IFNT treatment. Data are presented as mean \pm SEM. * P <0.05, ** P <0.001, *** P <0.0001.

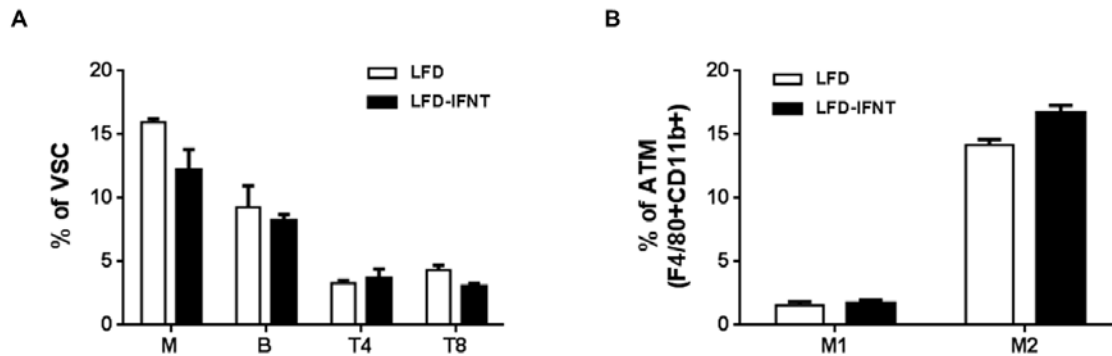


Figure 4.7 The infiltration of immune cells into adipose tissues of mice fed a low fat diet (LFD). A, Macrophage (M), B cells (B), CD4⁺ T cells (T4) and CD8⁺ T cells (T8) in visceral fat stromal cells (VSC) of visceral adipose tissues (VATs) of mice fed a LFD were not affected by treatment with IFNT based on results from flow cytometry analyses using antibodies against F4/80, CD11b, B220, CD4 and CD8. B, The M2 macrophage subtype VSC of VATs of mice fed a LFD was not affected by IFNT treatment based on flow cytometry analyses using antibodies against F4/80, CD11b, CD11c and CD206. Data are presented as mean \pm SEM (n=3-4).

Interferon tau modulates macrophage polarization

Given the distinct shift in activation status of adipose tissue macrophages *in vivo*, we further evaluated the effects of IFNT on macrophage polarization using a well-established *in vitro* model.¹⁵⁹ Bone marrow-derived macrophages were treated with IFNT at various dosage in the presence of LPS (100 ng/mL) for M1 activation or IL-4 (20 ng/mL) for M2 activation and activation of associated surface antigens was determined using flow cytometry assays. M2

macrophages (IL-4 treatment) displayed a significantly enhanced activation pattern as judged by stronger induction of surface markers CD69, CD80, and CD86 at 48 h after stimulation (Figure 4.8A). In contrast, activation of M1 macrophages induced by LPS treatment was significantly stalled in the presence of IFNT resulting in the left shift of surface marker levels (Figure 4.8B). We further examined the cytokine production profiles in these M1 and M2 macrophages using qRT-PCR analysis. As expected, IFNT significantly suppressed expression of proinflammatory cytokines IL-1 β and TNF- α by BMDMs in response to LPS stimulation compared to control BMDMs (Figure 4.8C, D). In addition, cells also displayed a slight increase in IL-10 upon IL-4 stimulation in the presence of IFNT (Figure 4.8E). PPAR γ is a key regulator that suppresses proinflammatory M1 and promotes anti-inflammatory M2 activation. Interestingly, IFNT did not affect IL-4-dependent PPAR γ expression in M2 macrophages, but IFNT significantly increased PPAR γ expression in M1 macrophages (Figure 4.8F), which suggests a potent inflammatory suppressing impact of IFNT on macrophage polarization.

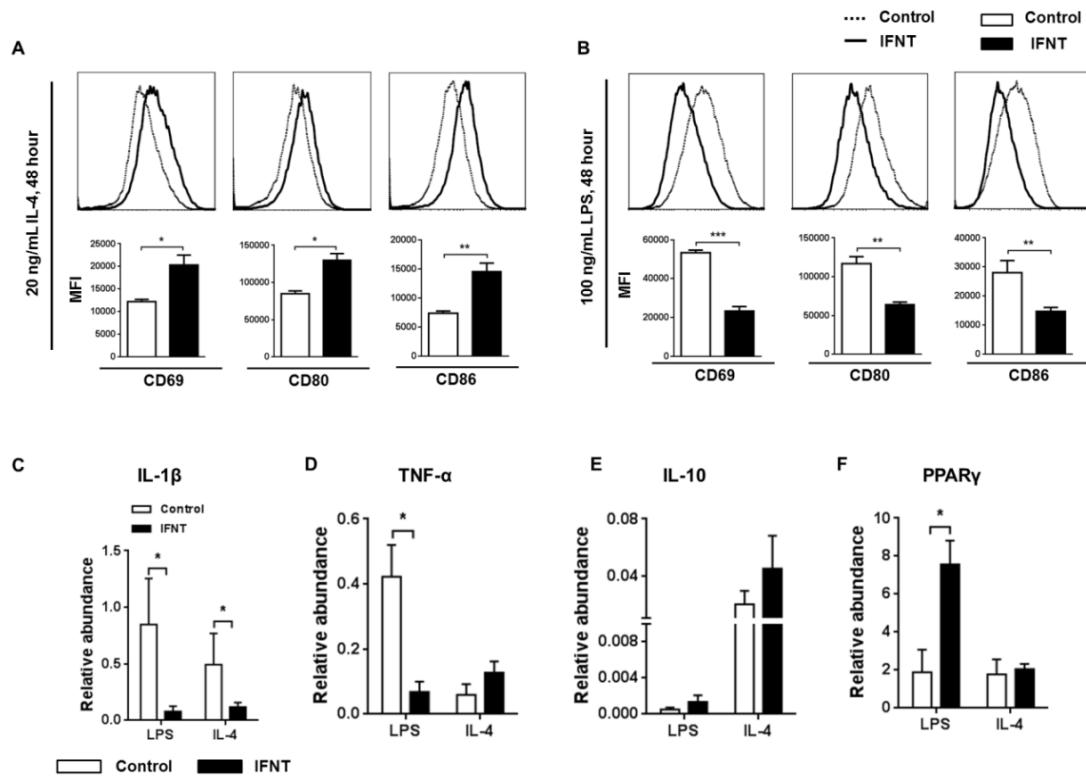


Figure 4.8 Interferon tau (IFNT) modulates macrophage polarization and cytokine profiles. The activation-related surface makers CD69, CD80, and CD86 of bone marrow derived macrophages (BMDM) were analyzed using flow cytometry after 48-h treatment with interleukin 4 (IL-4, 20 ng/mL; A) or lipopolysaccharide (LPS, 100 ng/mL; B) (n=3). The BMDMs were treated with IFNT at 5,000 antiviral units (AVU)/mL. The expression of cytokines IL-1 β (C), TNF- α (D) and IL-10 (E) and peroxisome proliferator-activated receptor γ (PPAR γ) (F) in BMDMs activated in the presence of IFNT (black bars) were analyzed by qRT-PCR (normalized to β -actin, n=3) and compared to activated BMDMs with no IFNT treatment (white bars). The results indicated that IFNT treatment promotes M2 activation but suppresses M1 activation of macrophages. Data are presented as mean \pm SEM. * P <0.05, ** P <0.001, *** P <0.0001. MFI, medium fluorescence intensity.

To understand the mechanism of IFNT action in regulating macrophage polarization, we examined signaling pathways mediated by type I interferon receptors in activated macrophages. We found that the expression of type I interferon receptor (IFNAR) was not affected by IFNT treatment in activated BMDMs (Figure 4.9). However, IFNT significantly enhanced STAT3 activation upon IL-4 stimulation as evidenced by significantly elevated phosphorylated STAT3 protein detected by intracellular staining assays followed by flow cytometric analysis (Figure 4.10A). Alternatively, type I interferons can exert anti-inflammatory functions through inducing activation of interferon-stimulated gene factor-3 (ISGF3) complex, which includes STAT1, STAT2, and interferon regulatory factor 9 (IRF9). In M2 macrophages, activation of STAT1 was increased by IFNT compared to the control (Figure 4.10B). In LPS-stimulated BMDMs, IFNT did not affect the phosphorylation status of STAT1 or STAT3 (Figure 4.10A, B). In addition, the abundance of IRF9 was significantly decreased in M1 macrophages, but increased in M2 macrophages in response to IFNT (Figure 4.10C). Thus, these results suggest that IFNT may modulate macrophage polarization primarily through controlling activation of ISGF3 complex and STAT3 pathway.

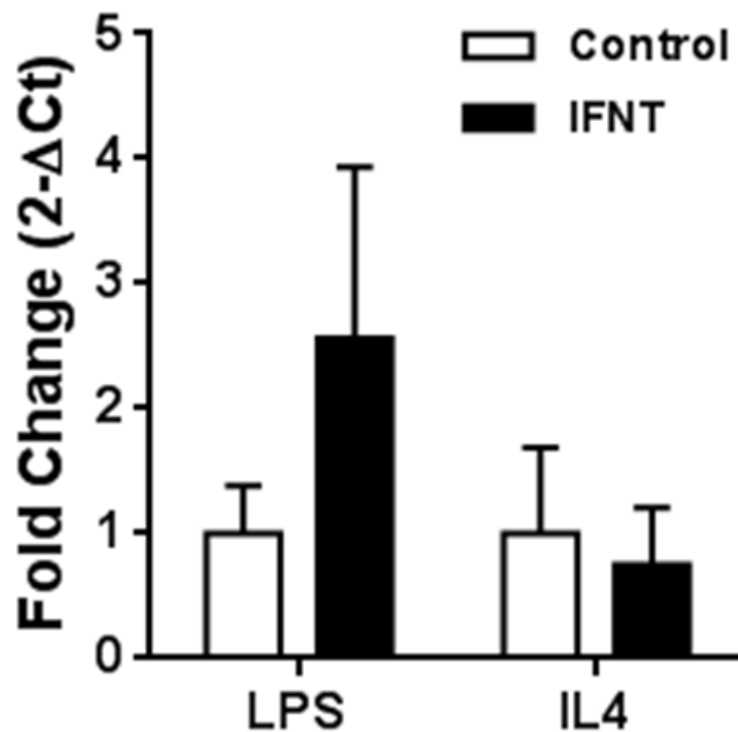


Figure 4.9 Effect of interferon tau (IFNT) on expression of type I interferon receptor in bone marrow derived macrophages (BMDMs). The abundance of type I interferon receptor in BMDM was not affected by IFNT based on results of qRT-PCR analysis after treatment for 48-hours with lipopolysaccharide (LPS, 100 ng/mL) or interleukin 4 (IL4, 20 ng/mL; n=3) with or without IFNT at 5,000 AVU/mL. Data are presented as mean \pm SEM.

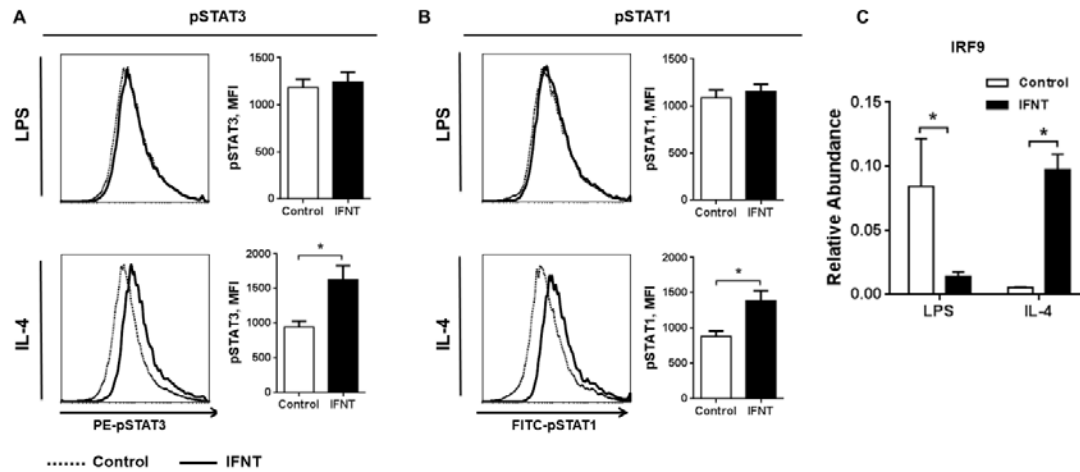


Figure 4.10 Interferon tau (IFNT) induces activation of STAT1 and STAT3 in bone marrow derived macrophages (BMDMs). A and B, The phosphorylation of signal transducer and activator of transcription 3 (pSTAT3) and pSTAT1 in BMDMs was increased in response to IFNT as measured by flow cytometry after 90-min of lipopolysaccharide (LPS, 100 ng/mL) or interleukin 4 (IL-4, 20 ng/mL) stimulation (n=3). C, The expression of interferon regulatory factor 9 (IRF9) was inhibited by IFNT after cells were exposed for 48-h to LPS, but increased in cells after 48-h stimulation by IL4 (n=3). Data are presented as mean \pm SEM. * $P < 0.05$, ** $P < 0.001$.

Discussion

Adipose tissue inflammation is a major contributor to the pathogenesis of obesity-associated insulin resistance.^{5,80,139} Using a diet-induced obesity model, we observed a significant increase in inflammation in both adipose tissue and in the systemic circulation of obese mice that was accompanied by exacerbated insulin resistance. This is consistent with previous reports and confirms the direct correlation between tissue inflammation with obesity-associated insulin resistance. Thus, results of our research provided a new set of evidence to support the development of therapeutic strategies with anti-inflammatory agents to treat patients with obesity-induced insulin resistance and subsequent type 2 diabetes. Type I interferons exert profound anti-inflammatory effects through modulating immune cell functions.²¹⁸ However, patients treated with IFNA and IFNB, two of the available interferon treatment options, often display severe high fever, as well as damage to liver and kidney functions.^{221,222,228} Such adverse effects greatly inhibit the adoption of type 1 interferons that may have beneficial effects. Compared to IFNA and IFNB, IFNT displays similar immunomodulatory functions, but it has low cytotoxicity even at higher dosages, and can provide a new option to treat obesity-induced insulin resistance and autoimmune disorders.^{134,224}

Type I interferons share high similarities among their members in both primary nucleotide coding and protein folding structure.²²³ Structural analysis

demonstrated that IFNT binds type I interferon receptors to activate type I interferon intracellular signaling pathways that alter immune status.²²⁹ This was confirmed in our study using an *in vitro* culture system. IFNT-treated BMDMs displayed a significantly suppressed inflammatory response to LPS accompanied by decreased production of IL-1 β and TNF- α . This effect is partially mediated by suppressing the ISGF3 pathway. In addition, IFNT significantly enhanced the anti-inflammatory response, namely activation of M2 macrophages. Accordingly, decreased expression of IL-1 β was observed in BMDMs stimulated with IL-4 in the presence of IFNT. Our *in vivo* results further confirmed the anti-inflammatory effects of IFNT in adipose tissues, as evidenced by suppression on inflammatory pathways and production of proinflammatory cytokines.

Given the potent anti-inflammatory effects of IFNT *in vivo*, it is not surprising that IFNT ameliorated insulin resistance in the obese mice. IFNT administration did not affect body weight gain or adiposity of obese mice, or metabolic status of adipose tissues and liver of obese mice (Figure 4.11), but there was a decrease in concentrations of triglycerides in plasma from HFD-IFNT mice (Figure 4.12). These results are not consistent with results of a previous study with Zucker diabetic fatty rats (ZDF) in which oral IFNT reduced white fat mass; however, the results of the present study and that with the ZDF rats are consistent in that IFNT enhanced the anti-inflammatory state in both models.²²⁷ The lack of effect of IFNT on white fat mass may be due to

differences in animal models and length of IFNT treatment as the ZDF rats were treated from 4 to 12 weeks of age. Intriguingly, the composition of infiltrated immune cells, especially the macrophage subtypes, in adipose tissues was significantly altered upon IFNT treatment. The overall adipose tissue immune cells including T cell, B cell and macrophage populations in the mice treated with IFNT were comparable to the control mice, suggesting that IFNT treatment did not affect immune cell recruitment, which was concomitant with unchanged expression of CCL2 in adipose tissues from IFNT-treated HFD and control mice. However, adipose tissue inflammation was significantly suppressed by IFNT treatment as evidenced by decreased expression of proinflammatory cytokines, which was associated with increased Akt activation upon insulin stimulation. This is partially attributed to the shift of adipose tissue macrophage status resulting in more M2 than M1 macrophages. The M2 macrophages exert anti-inflammatory effects in the tissue microenvironment including regulation through increased secretion of anti-inflammatory cytokines, including IL-10.⁶⁶ We observed greater abundance of IL-10 in HFD mice treated with IFNT. Results of a previous study suggest that type I interferons act through either the ISGF3 complex or STAT3 in immune cells.^{218,219} Our results suggested that IFNT functions through the ISGF3 complex in macrophages and also has a significant effect to increase STAT3 phosphorylation, resulting in significantly enhanced production of IL-10. Further understanding of the mechanism of IFNT action in mitigating obesity-

associated symptoms and its potential impact on other immune cell activation await further investigations.

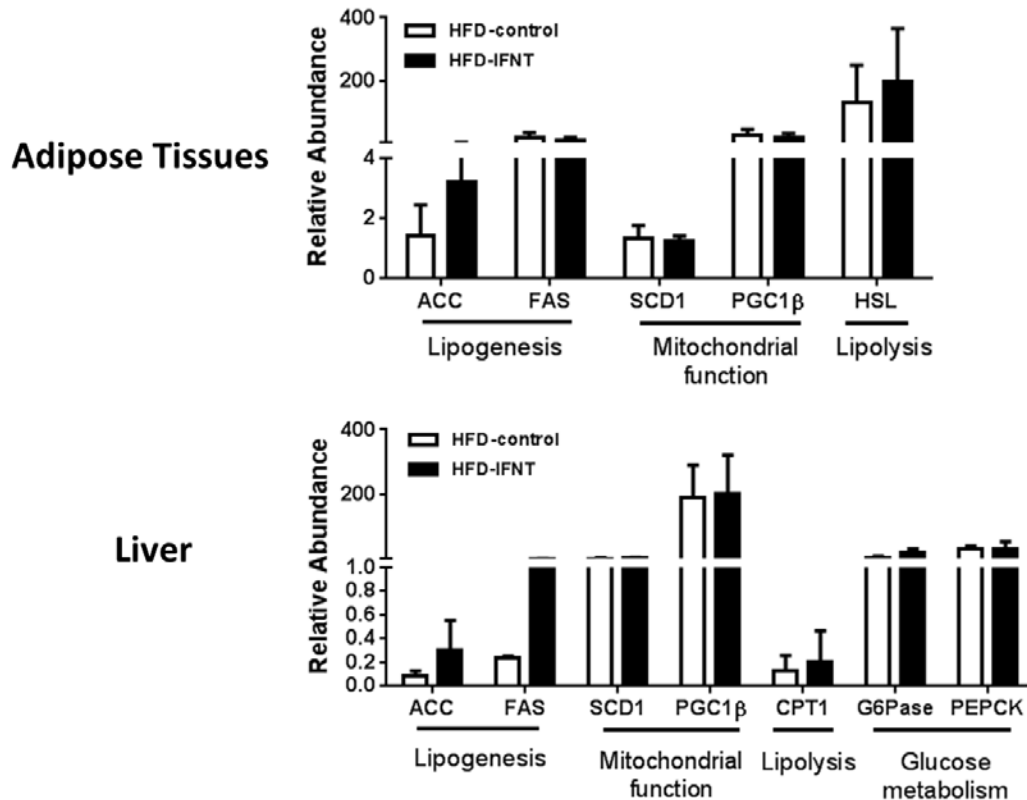


Figure 4.11 The effects of interferon tau (IFNT) on lipogenesis, mitochondrial, or lipolysis of adipose tissues and liver of mice fed a high fat diet (HFD). The expression of key regulators for lipogenesis, mitochondrial function, lipolysis was measured in the adipose tissues (upper panel) and liver (lower panel) collected from mice fed a HFD mice or a HFD and treated with IFNT mice was not affected by IFNT based on qRT-PCR analyses normalized to β -actin. Data are means \pm SEM, $n=3$. ACC, acetyl-CoA carboxylase; FAS, fatty acid synthetase; SCD1, stearoyl-CoA desaturase-1; PGC1 β , peroxisome proliferator-activated receptor gamma, coactivator 1 beta; CPT1, carnitine palmitoyltransferase 1; HSL, hormone-sensitive lipase; G6pase, glucose 6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase.

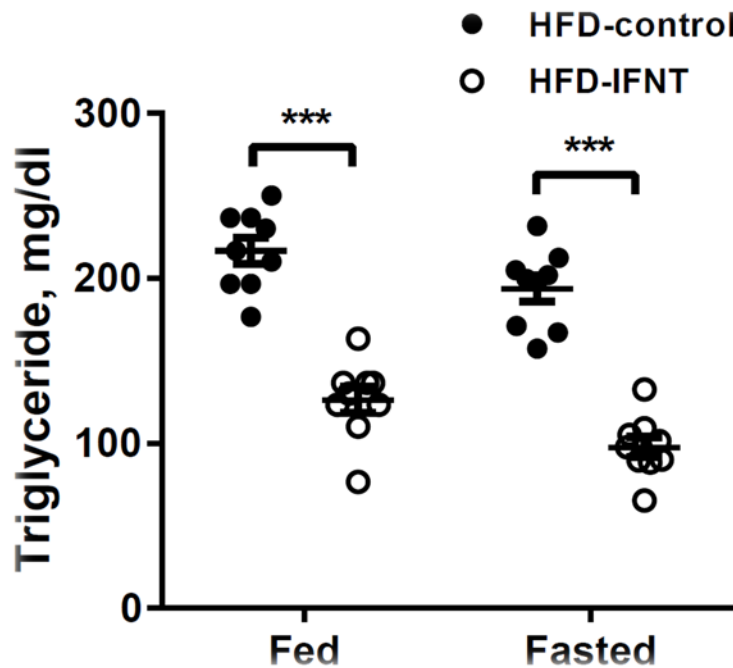


Figure 4.12 The effect of interferon tau (IFNT) on concentration of triglyceride in plasma of mice fed a high fat diet (HFD). Concentrations of triglyceride in plasma were decreased ($P < 0.01$) in mice on a HFD that were treated with IFNT or fasted for 16 h. Data are presented as mean \pm SEM.

In summary, results of our study demonstrate that IFNT is a potent regulator for obesity-associated insulin resistance and tissue inflammation that is accounted for partially by effects of IFNT to control adipose tissue macrophage polarization. Given the low cytotoxicity of IFNT compared to other members of the type I interferon family, results of our study provide the first evidence to support the potential application of IFNT to mitigate obesity-associated syndromes including various autoimmune diseases.

CHAPTER V

SUMMARY

Chronic low grade inflammation in adipose tissue of obese individuals is a causal factor in the pathogenesis of obesity-associated diseases, including type 2 diabetes and cardiovascular diseases. Dramatic increases in immune cells in visceral adipose tissues of obese individuals primarily display proinflammatory profiles characterized by elevated concentrations of inflammatory cytokines and systemic insulin resistance. However, the mechanisms regulating functions of immune cells in adipose tissue niches in response to obesity are poorly understood.

During the development of obesity, B cells become a major immune cell component in visceral fat pad and exert profound impacts on the adipose tissue functions. In this thesis study, I have demonstrated that miR-150, a B cell specific regulatory microRNA, is a critical modulator of the pathogenesis of chronic tissue inflammation and insulin resistance associated with obesity, which are major contributors to metabolic syndrome and development of type 2 diabetes and other cardiovascular complications. miR-150 controls B cell functions and their cell-cell interaction with other immune cells through simultaneous targeting of multiple targets *Elk1*, *Etf1*, and *Myb*. These miR-150 target genes can exert a potent impact on the BCR signaling network governing adipose tissue B cell function, which can, in turn, change the adipose tissue

niche and its function. Further investigations regarding the mechanisms of action of miR-150 and B cells will provide important information toward development of therapeutic strategies to alter microRNAs and modulate B cells to treat obesity-associated complications.

In the adipose tissue, the composition of ATMs with polarized activation status is critical for maintaining adipose tissues homeostasis. Thus, it is critical to understand the mechanisms underlying the activation of ATMs. In this thesis study, I have showed that the PPAR γ -miR-223 regulatory axis is critical for modulating the activation status of ATMs and subsequent adipose tissue inflammation and insulin sensitivity. miR-223 is a PPAR γ -induced genes during the M2 responses. miR-223 and its network involving target genes *Rasa1* and *Nfat5* act as the critical downstream components of the PPAR γ -mediated cell signaling pathway that regulates activation of adipose tissue macrophages. Furthermore, identification of novel miR-223 target genes *Nfat5* and *Rasa1* in this context provides new gene targets for development of drugs with the potential to mitigate obesity-related diseases.

The results of last study demonstrate that interferon tau is a potent regulator for obesity-associated insulin resistance and tissue inflammation that is accounted for partially by effects of interferon tau to control adipose tissue macrophage polarization. Further study demonstrates that IFNT functions through the ISGF3 complex in macrophages and also has a significant effect to

increase STAT3 phosphorylation, resulting enhanced M2 responses. Given the low cytotoxicity of interferon tau compared to other members of the type I interferon family, results of this study provide the first evidence to support the potential application of interferon tau to mitigate obesity-associated syndromes including various autoimmune diseases.

Collectively, the findings from these thesis studies will open a window to the development of novel therapeutic strategies to mitigate obesity-related diseases.

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APPENDIX

Table A-1 High-fat diet formula

Nutrient	%	kcal%
Protein	26.2	20
Carbohydrate	26.3	20
Fat	34.9	60
Total kcal/gm	5.24	100
Ingredient	gram	kcal
Casein, 30 mesh	200	800
L-cystine	3	12
Maltodextrin 10	125	500
Sucrose	68.8	275.2
Cellulose, BW200	50	0
Soybean oil	25	225
Lard*	245	2205
Mineral mix S10026	10	0
Dicalcium phosphate	13	0
Calcium carbonate	5.5	0
Potassium citrate, H ₂ O	16.5	0
Vitamin mix V10001	10	40
Choline bitartrate	2	0
FD&C Blue Dye#1	0.05	0
Total, gram	773.85	4057

*Typical analysis of cholesterol in lard = 0.72 mg/gram

Cholesterol (mg)/4057 kcal = 216.4

Cholesterol (mg)/kg = 279.6

Table A-2 Low-fat diet formula

Nutrient	%	kcal%
Protein	19.2	20
Carbohydrate	67.3	70
Fat	4.3	10
Total kcal/g	3.85	100
Ingredient	gram	kcal
Casein, 30 mesh	200	800
L-cystine	3	12
Corn starch	315	1260
Maltodextrin 10	35	140
Sucrose	350	1400
Cellulose, BW200	50	0
Soybean oil	25	225
Lard*	20	180
Mineral mix S10026	10	0
Dicalcium phosphate	13	0
Calcium carbonate	5.5	0
Potassium citrate, H ₂ O	16.5	0
Vitamin mix V10001	10	40
Choline bitartrate	2	0
FD&C Blue Dye#1	0.05	0
Total, gram	1055.05	4057

*Typical analysis of cholesterol in lard = 0.72 mg/gram

Cholesterol (mg)/4057 kcal = 54.4

Cholesterol (mg)/kg = 51.6

Table A-3 Adipose tissue digestion buffer preparation

	concentration
HBSS	1x
HEPES	20mM
P/S	0.1 KU/mL
BSA	0.015 g/mL
Collagenase II	2 mg/mL

HBSS, Hank's Balanced Salt Solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; P/S, penicillin-streptomycin; BSA, bovine serum albumin.

Table A-4 Red blood cell lysis buffer

NH ₄ Cl	8.3 gram
KHCO ₃	1 gram
EDTA	37 milligram
ddH ₂ O	1000 mL

Table A-5 Solutions for chromatin immunoprecipitation assay

Formaldehyde Solution	Stock buffer	50ml
50 mM HEPES-KOH pH 7.5	1 M HEPES-KOH pH 7.5	2.5
Or 50 mM HEPES native	Or 1 M HEPES	
100 mM NaCl	5 M NaCl	1
1 mM EDTA	500 mM EDTA	0.1
0.5 mM EGTA	500 mM EGTA	0.05
37% formaldehyde		1.375
ddH ₂ O		44.975
Lysis Buffer 1	Stock buffer	50ml
50 mM HEPES-KOH pH 7.5	1 M HEPES-KOH pH 7.5	2.5
140 mM NaCl	5 M NaCl	1.4
1 mM EDTA	500 mM EDTA	0.1
10% glycerol	50% glycerol	5
0.5% NP-40	10% NP-40	2.5
0.25% Triton X-100	10% Triton X-100	1.25
1× protease inhibitors	100X PI	0.5
0.02 M Na-Butyrate	2 M Na-Butyrate	0.5
ddH ₂ O		37.25
Lysis Buffer 2	Stock buffer	50ml
10 mM Tris-HCl, pH 8.0,	1 M Tris-HCl, pH 8.0	0.5
200 mM NaCl	5 M NaCl	2
1 mM EDTA	500 mM EDTA	0.1
0.5 mM EGTA	500 mM EGTA	0.05
1× protease inhibitors	100X PI	0.5
0.02 M Na-Butyrate	2 M Na-Butyrate	0.5
ddH ₂ O		47.35
Lysis Buffer 3	Stock buffer	50ml
10 mM Tris-HCl, pH 8.0	1 M Tris-HCl, pH 8.0	0.5
100 mM NaCl	5 M NaCl	1
1 mM EDTA	500 mM EDTA	0.1
0.5 mM EGTA	500 mM EGTA	0.05
0.1% Na-Deoxycholate	5% Na-Deoxycholate	1
0.5% N-lauroylsarcosine	5% N-lauroylsarcosine	5
1× protease inhibitors	100X PI	0.5
0.02 M Na-Butyrate	2 M Na-Butyrate	0.5
ddH ₂ O		42.35

Table A-5 Continued

Elution Buffer	Stock buffer	50ml
50 mM Tris-HCl, pH 8.0	1 M Tris-HCl, pH 8.0	2.5
10 mM EDTA	500 mM EDTA	1
1.0% SDS	10% SDS	5
ddH ₂ O		41.5
TE + NaCl:	Stock buffer	50ml
10 mM Tris-HCl pH 8.0	1 M Tris-HCl, pH 8.0	0.5
1 mM EDTA	500 mM EDTA	0.1
50 mM NaCl	5 M NaCl	0.5
ddH ₂ O		48.9
TBS	Stock buffer	50ml
150 mM NaCl	5 M NaCl	1.5
10 mM Tris pH8.0	1 M Tris-HCl, pH 8.0	0.5
1× protease inhibitors		0.5
0.02 M Na-Butyrate	2 M Na-Butyrate	0.5
ddH ₂ O		48
Elution Buffer ChIP	Stock buffer	50ml
50 mM Tris-HCl pH8.0	1 M Tris-HCl, pH 8.0	2.5
10 mM EDTA	500 mM EDTA	1
1.0% SDS	10% SDS	5
ddH ₂ O		41.5
Low-Salt wash buffer	Stock buffer	50ml
20 mM Tris-HCl, pH 8.0,	1 M Tris-HCl, pH 8.0	1
150 mM NaCl	5 M NaCl	1.5
1 mM EDTA	500 mM EDTA	0.1
0.5 mM EGTA	500 mM EGTA	0.05
0.1% SDS	10% SDS	0.5
1% Triton X-100	10% Triton X-100	5
ddH ₂ O		41.85

Table A-5 Continued

High-Salt wash buffer	Stock buffer	50ml
20 mM Tris-HCl, pH 8.0,	1 M Tris-HCl, pH 8.0	1
500 mM NaCl	5 M NaCl	5
1 mM EDTA	500 mM EDTA	0.1
0.5 mM EGTA	500 mM EGTA	0.05
0.1% SDS	10% SDS	0.5
1% Triton X-100	10% Triton X-100	5
ddH ₂ O		38.35
LiCl wash buffer	Stock buffer	50ml
10 mM Tris-HCl, pH 8.0,	1 M Tris-HCl, pH 8.0	0.5
0.25M LiCl	5 M LiCl	2.5
1 mM EDTA	500 mM EDTA	0.1
0.5 mM EGTA	500 mM EGTA	0.05
1% Na-Deoxycholate	5% Na-Deoxycholate	10
1.0% NP-40	10% NP-40	5
ddH ₂ O		31.85